STUDIES ON THE BIODETERIORATION OF POLYURETHANES BY FUNGI

by

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Being a thesis submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy

University of Aston in Birmingham

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SUMMARY

The work reported in this thesis was carried out to increase knowledge of the mechanisms by which fungi deteriorate polyurethanes and the conditions which encourage this to take place.

Five fungi, Gliocladium roseum, Chaetomium globosum, Penicillium citrinum, Aspergillus fumigatus and Nigrospora spherica, were selected as potential biodeteriogens of polyurethanes from initial screening experiments. This list included three species which had not been previously reported as polyurethane deteriogens. Their response to temperature, pH and their modes of attack on polyurethanes were studied. The results indicated that exoenzymes were the major cause of the biodeterioration which took place, and therefore, protease, esterase and urease activities of the fungi were studied using modifications of Rautela and Cowling's test tube method.

A comparison of enzyme activities, among the test fungi and their respective degradation rates was made in order to study the relationship between enzymes and biodeterioration. For this purpose, percent weight losses, changes in tensile strengths and percent ultimate elongations of four polyester polyurethane and one polyether polyurethane elastomers were used.

The results indicated that 'polyurethane degrading' enzymes are inducible and very closely resemble esterases. The susceptibility of polyester types and the resistance of polyether types, in conjunction with the results from chemical and spectral analysis of degraded products supported these findings. The investigation suggested that the splitting of the molecular chains by the 'polyurethane degrading' enzyme systems took place probably via two paths, i.e. random splitting and regular splitting.

This study has increased knowledge of the fungal deterioration of polyurethanes by establishing the contribution of esterases in the breakdown process and the physical and mechanical changes which accompany this.

KEY WORDS

fungi : polyurethane : biodeterioration : enzymes : polyester

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to my wife, Radha, and

my daughter, Supun.

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CHAPTER ONE

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Polyurethanes are a relatively new class of man-made polymers that have found widespread commercial application since World War II. This group of polymers is so versatile that probably no other polymer or a group of polymers is used in so wide a range of applications, to suit so many environments.

The term "polyurethane" is more one of convenience than of accuracy, since they are not derived by polymerizing a monomeric urethane molecule, nor are they usually polymers which contain a significant number of urethane groups regardless of what the rest of the molecule may be. Other chemical groups which may appear in the structure are ester, ether, urea, amine, aromatic, amide, biuret and allophanate along with the hydrocarbon groups. The manner in which these groups are put together and at which group they are branched will determine to a large extent the major physical and chemical properties of the finished polymer. The easily varied chemical and physical properties make them ideal for many commercial and industrial applications. Some of the uses of this diverse polymer include foams, elastomers, poromerics, paints, fibres, fabric coatings, adhesives and sealants.

The market for polyurethanes in the U.K. has been increasing steadily up to 1979. "The U.K. Plastics Industry - 1979" (1980) showed that polyurethane consumption

in the United Kingdom has increased to 94,500 tons from 89,000 tons in 1978. But due to deep recession the sales dropped sharply and the consumption for 1980 was reported to be 84,000 tons. "The U.K. Plastics Industry - 1981" (1982) showed that the polyurethane market in the U.K. remained fairly static at 83,500 tons in 1981 even with a prolonged recessionary period. It forecasted an increase in polyurethane consumption in the U.K. in 1982 with soft polyurethane showing a 2% increase and hard polyurethanes a 7% increase.

1.1 Raw materials and their reactions.

The versatility of polyurethane polymers is associated with a greater than normal complexity in manufacture. Indeed the manufacture of polyurethanes involves a greater degree of control of chemical reactions than most other polymer manufactures. Therefore a knowledge of the chemical background is essential in order to understand fully the process of microbial breakdown of these polymers.

The main raw materials used in the production of polyurethanes are isocyanates and active hydrogen compounds such as alcohols, amines, carboxylic acids, ureas, amides etc.. The reactions of isocyanates with these active hydrogen compounds (Johnson, 1968) can be summed up as follows:-

a. $R.NCO$(isocyanate) + R^1H (hydrocarbon) $\rightarrow$ $R.NH.CO.R^1$(amide)

b. $R.NCO + R^1.OH$ (alcohol) $\rightarrow R.NH.CO.OR^1$ (urethane)

c. $R.NCO + R^1.NH_2$ (amine) $\rightarrow R.NH.CO.NH.R^1$ (urea)

d. $R.NCO + H.OH$ (water) $\rightarrow R.NH.COOH$ (carbamic acid)
$\downarrow$
$R.NH_2 + CO_2\uparrow$
$\downarrow$ R.NCO
$R.NH.CO.NH.R$ (urea)

e. $R.NCO + R^1.COOH$ (carboxylic acid) $\rightarrow R.NH.CO.O.CO.R^1$
(mixed acid anhydride)
$\downarrow$
$R.NH.CO.R^1 + CO_2$
(amide)

These reactions are the backbone of polyurethane chemistry. In addition, isocyanates undergo further analgous reactions with the groups containing active hydrogen which are present in all the primary products of the reactions mentioned above, as follows:-

f. $R.NCO + -NH.CO.O-$ (urethane) $\rightarrow -N(R.NH.CO)-CO.O-$ (allophanate)

g. $R.NCO + -NH.CO.NH-$ (urea) $\rightarrow -N(R.NH.CO)-CO.NH-$ (Biuret)

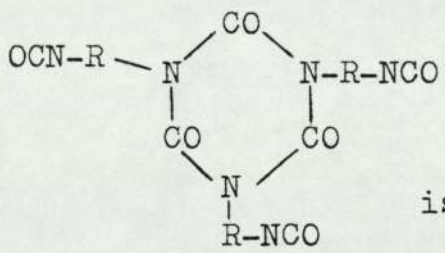
h. $R.NCO + -NH.CO-$ (amide) $\rightarrow -N(R.NH.CO)-CO-$ (acyl urea)

These secondary reactions occur to a much lesser extent than the primary reactions, but, their importance must not

be under-estimated; the formation of allophanates and particularly biurets is responsible for cross-linking and branching which has an important effect on polyurethane properties in many instances.

Reactions of isocyanate comprise the polymerization reactions and are also of some importance in influencing branching and cross-linking during the build up of polyurethane structure. The most important reaction is the formation of the isocyanurate ring. It is formed by trimerization of isocyanates and can be shown as follows:-

i. 3 OCN.R.NCO →



isocyanurate

Isocyanurates are exceptionally stable and because they contain many isocyanate groups they behave as multi functional isocyanates influencing branching and cross-linking.

A full understanding of the chemistry of addition reactions of isocyanates must take account of the stability of the reaction products. The primary and secondary addition reactions of isocyanates are all reversible, excluding of course the reactions with water and with carboxyl which are condensations rather than additions, involving the elimination of CO_2. The reaction products of isocyanates with ureas and urethanes, i.e. biurets and allophanates, dissociate more readily than the ureas and urethanes themselves.

As polyurethanes are primarily based on the reaction of isocyanates with polyesters and polyethers, it is worthwhile looking briefly at the formation of these basic polymeric structural units. The build up of polyesters by condensation of polyalcohols and dicarboxylic acids are illustrated schematically below. The use of excess glycol affords predominantly hydroxyl-ended groups although a few carboxyl-ended groups always persist. Glycols afford linear polyesters whilst use of higher polyhydric alcohols lead to branched polyesters.

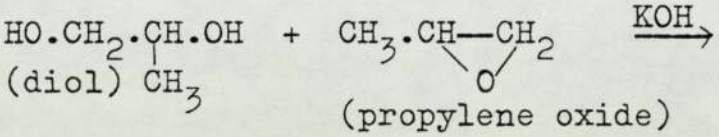
$$\underset{\text{(dicarboxylic acid)}}{HOOC.R.COOH} + \underset{\text{(diol)}}{HO.R^1.OH} \xrightarrow[-H_2O]{\Delta} \underset{\text{(hydroxyl-ended linear polyester)}}{HO.R^1.O(OC.R.CO.OR^1.O)_nH} + \underset{\text{(carboxyl/hydroxyl-ended linear polyester)}}{HO(OC.R.CO.OR^1.O)_nH}$$

$$HOOC.R.COOH + \underset{\text{(triol)}}{HO - \underset{\displaystyle OH}{\underset{|}{R^{11}}} - OH} + \underset{\text{(diol)}}{HO.R^1.OH} \xrightarrow[-H_2O]{\Delta}$$

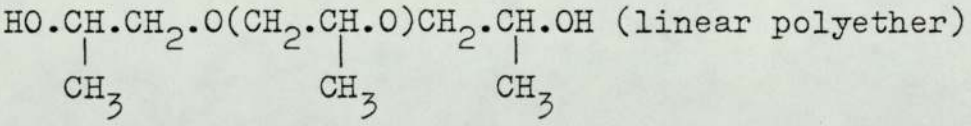
$$HO.R^1.O(OC.R.CO.OR^1.O.CO.R.CO.O-\underset{\displaystyle OH}{\underset{|}{R^{11}}}-O)_nH$$

(branched polyester)

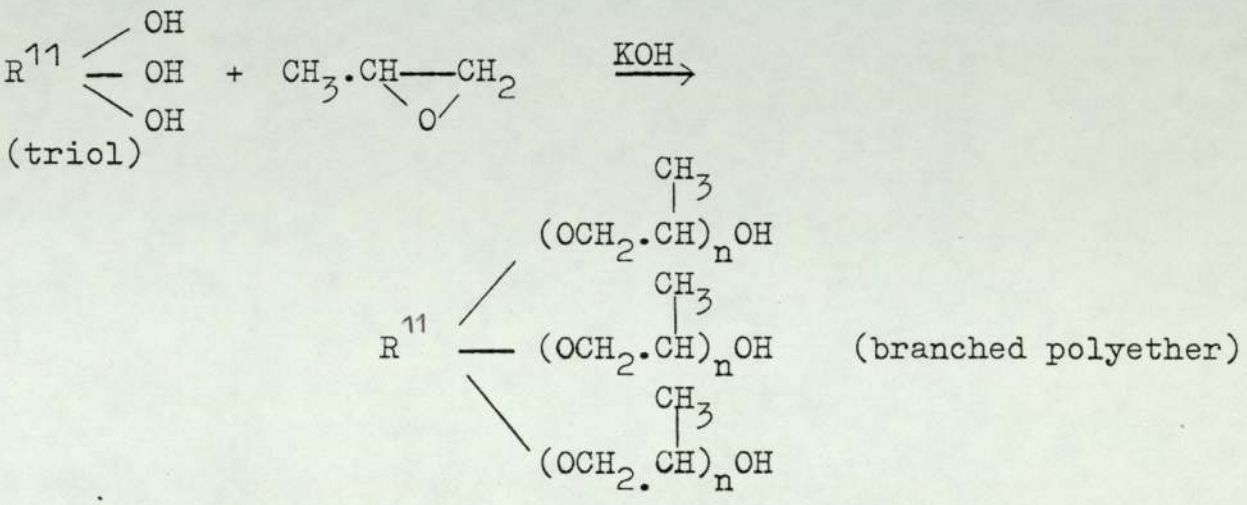
The polyethers most frequently used are base-catalysed polymers of propylene or ethylene oxides using a glycol as initiator when linear polyethers are required, and polyols of higher functionality (e.g. glycerol), to obtain branched products. This is illustrated schematically below:-



(diol) (propylene oxide)



(linear polyether)



(triol) (branched polyether)

In commercial practice the chemistry of polyurethanes involves far more than simply the reactions between the diisocyanates and the hydrogen donors. Many other materials are required in order to obtain a well balanced formulation for any specific end product with the required properties.

The choice of these additives is as important to the formulation, as is the choice of the proper diisocyanate and the proper hydrogen donors (Doyle, 1971). They are additives for catalysis, to promote or accelerate the reaction between major components of the formulation to ensure the complete reaction of all the components in the formulation.

There are additives which will protect the polymer against the elements of nature such as ultraviolet light,

ozone, oxidation and other hazards the polymer will encounter when exposed to weather. Some additives are used to control the flow of the polymer and some others to aid release of CO_2 from the polymer to prevent bubbles at the surface.

There are a great many pigments and fillers that may be used in polyurethanes to achieve proper colouration, to extend (and thus lower total cost) and to impart special properties to the polymer. Unlike other commercial plastic formulations, it is rare that polyurethane formulations will consist of more than half their weight of these low cost materials. The low cost additives used to plasticize and extend have little effect other than to soften and increase the elongation. There are other additives such as flame retardants which add appreciably to other properties of the polymer.

Many other additives, both liquid and solid, perform very special functions in the polymer. Some of them will take up moisture from the formulation. Others will aid in the control of gloss of the urethanes.

These indicate the importance of additives in polyurethane formulations even though they are used in relatively low quantities.

1.2 Classification of polyurethane structures

Polyurethanes may range in structure from polymers with a chain structure of regularly repeating urethane groups to

polymers in which the urethane groups merely link together other polymeric chains at irregular intervals, and from essentially linear to highly cross-linked network polymers. These polymers may be classified according to the structure of the polymer chain (Trappe, 1968) into four types, which may be differentiated as follows:-

1. Regular polyurethanes - Polymers with a chain structure of regularly repeating urethane groups. They are made by reaction of diisocyanates and monomeric diols.

2. Urethane-extended polyurethanes - Polymers containing relatively few urethane groups distributed in the polymer chain at irregular intervals and whose presence is consequent on the fact that the urethane forming reaction was used to link together another polymeric species. These are made by reacting polyisocyanates with polymers containing hydroxyl groups, so that the parent polymer chains become extended through the formation of urethane groups.

3. Copolymers with polyurethane segments - Polymers having a segmented or block structure comprising segments containing regular repeating urethane groups (regular polyurethanes) linked to polymeric segments of another type. Such copolymers are obtained when a diisocyanate is reacted with a combination of hydroxyl containing polymer and low molecular weight diol. Commercial interests in this type of polymer are largely as tough, moderately hard to very hard elastomers.

4. Urethane-linked segmented copolymers - Polymers having a segmented or block structure in which segments other than polyurethane segments are linked together through a urethane group. This type of polyurethanes may be formed either a) by linking together two or more performed polymers containing appropriate reactive groups by reaction with diisocyanate or b) by interaction of a diisocyanate with a combination of both a performed hydroxyl-containing polymer and a low molecular weight monomeric compound with isocyanate-reactive groups other than hydroxyl. Copolymers of this type are used in manufacture of water-blown polyurethane foams and some types of solid polyurethane elastomers.

Although all the above types of chain structures can be discerned in polymers coming within the designation of polyurethane, the boundries between the types are not clearly definable since gradual transitions between types are possible. In the areas of transition the emphasis given to the various aspects of structure may depend upon the particular property under consideration.

1.3 Environmental properties

1.3.1 Resistance to weathering

The oxygen and ozone resistance of urethane elastomers are excellent because of the absence of olefinic bonds. The oxygen absorption of a polyester urethane has been found to be quite low (Seeger et al., 1953) and oven-aging has little

effect compared to natural rubber (Dinsmore, 1953).

Discolouration due to oxidation may occur (some formulations of the aromatic variety will tend to turn yellow and others brown) but it is not always an indication of a change in mechanical properties (Saunders and Frisch, 1964).

The most detailed study of weathering was done using Estane, a polyester polyurethane elastomer. Experimental evidence (Schollenberger et al., 1958 and 1961) showed that weathering of Estane involved an ultraviolet promoted autoxidation. Addition of certain amine and phenolic antioxidants was helpful in improving the weather resistance of Estane. Likewise, the incorporation of carbon black and ultraviolet absorbers such as Uvinul 490, proved to be most effective in outdoor exposure tests. This observation was further supported by Satas (1963), who showed a typical improvement on weathering by introducing antioxidants and ultraviolet absorbers into Estane formulation.

1.3.2 Resistance to gamma radiation

Polyurethanes are known to be quite resistant to gamma radiation. Harrington (1957) studied the effects of gamma radiation on Adiprene C as well as other polyurethane elastomers and found that these were superior to other elastomers studied including silicones and fluoroelastomers. The mechanical characteristics of Estane elastomer when exposed to gamma radiation did not appreciably change even up to 100 Mr (Harrington, 1959[a]; Schollenberger et al., 1960).

1.3.3 Resistance to chemicals

Urethane elastomers are resistant to attack by many chemicals (Saunders and Frisch, 1964). They exhibit excellent resistance towards aliphatic solvents, ether, most petroleum-based fuels and oils, hydraulic fluids and others. Swelling occurs in aromatic hydrocarbons (moderate), esters, ketones and degradation in strong or concentrated acids and bases and very strong oxidizing agents. Some polyurethane elastomers may be dissolved in dimethyl formamide containing a trace of dibutyl amine.

1.3.4 Resistance to temperature

1.3.4.1 High temperature

The effect of heat on polyurethanes has received considerable attention. Bunn (1955) showed that hydrogen bonding between the urethane chains accounted for the good mechanical properties of polyurethane elastomers. However, the same hydrogen bonding decreases with increasing temperature and this causes the loss of properties of polyurethanes with increasing temperatures.

Berger (1965) reported that urethane groups begin to dissociate at 150 - 200°C, while dissociation of allophanate and biuret groups occurs at lower temperatures. The urea groups dissociate above 200°C; the ether bonds oxidize and cleave at 250°C and the ester bonds are susceptible to hydrolysis.

Therefore the high temperature resistance of polyurethane elastomers depends on the type of the polyurethane used and on the service conditions. For continuous use for moderate times in air, the upper temperature limits were from about 88°C to 104°C (Saunders and Frisch, 1964). The harder grades retain their properties better than the softer grades. The polyester based elastomers have shown slightly higher temperature limit than the polyether based elastomers.

1.3.4.2 Low temperature

Many polyurethane elastomers possess very good low temperature properties (Saunders and Frisch, 1964). While gradual stiffening takes place at low temperatures certain polyether and polyester polyurethane elastomers do not become brittle at temperatures as low as -62°C to -71°C. Some elastomers, exhibiting greater symmetry in their molecular structure, may undergo crystallisation when held at moderately low temperatures for some period of time.

1.3.5 Resistance to the activity of water

Water absorbed by a polyurethane produces two effects, a reversible "plasticization" and irreversible "degradation" (Athey, 1965).

Plasticization results in a slight reduction in hardness and modulus and occurs presumably when the absorbed water forms hydrogen bonds with polar structures in the polymer chains and reduces the intermolecular attraction between

adjacent polymer molecules. The original properties are restored when the absorbed water is removed if significant chemical degradation of the polymer molecules has not occurred.

Hydrolytic degradation of the polymer chains cause a permanent reduction in the properties of the polymer. Hydrolysis of functional groups such as ester, urethane, urea, biuret and allophanate cause both chain scission and loss of branching and cross-linking.

Athey (1965) studied the changes in tensile strength due to plasticization and degradation of different polyester and polyether polyurethanes by absorbed water. He observed that the moisture content of the polyurethane vulcanizate at equilibrium with the atmosphere varied about two percent as the relative humidity changed from one hundred percent to zero percent. This change in moisture content caused only a small variation in vulcanizate properties which was slightly greater for polyethers than for polyesters.

He studied the degradation by absorbed water by immersing polyurethane pieces in distilled water at 50°C, 70°C and 100°C and also exposing the samples at 25°C to 100% relative humidity and at 70°C to 80% relative humidity and measuring the changes in tensile strength at different intervals. His results indicated that polyether-based polyurethanes were inherently more resistant to hydrolysis than polyester-based polyurethanes by a factor of 5-10. The rate of degradation of the polyester-based polyurethanes studied was independent of the diisocyanate or curing system used, indicating that

the ester group was the least resistant group in the molecule to hydrolysis. By contrast, the curing system used for the polyether-based polyurethanes had a significant influence on hydrolytic stability and glycol curing agents yielded the most resistant vulcanizates. This indicated that the ether group was the most resistant group in the polyether-based polyurethanes.

Most polyurethane elastomers prepared to date are not resistant to boiling water and other strong hydrolytic media for long periods of time. Harrington (1959) found that boiling for 1000 hours resulted in a loss of twentyseven percent to one hundred percent in the hardness of a variety of polyurethane types. The data also indicated that polyether-based elastomers were more resistant to hydrolysis than polyester-based elastomers. Seeger et al. (1953) also observed that the polyester polyurethane, Chemigum SL, showed poor resistance to hot water and steam.

1.4 Resistance to microbial attack

Unlike most plastics polyurethanes have frequently been found to be subjected to attack by microorganisms, especially fungi. There is considerable presumptive evidence that the polyester type of polyurethanes are commonly attacked by fungi, whereas polyethers may be completely or almost completely, resistant.

In 1962, the Wyandotte Chemical Corporation tested polyether sealing compounds for clay or concrete sewer pipes,

drain pipes or other underground service. Five polyether polyurethanes were used and they were subjected to a number of biological test procedures including incubation in broth medium and soil burial for 3 - 4 weeks. These tests had presumptively indicated resistance of these polyethers to microbial deterioration.

Osserfort and Testroet (1966) investigated polyester and polyether type polyurethane rubbers and found that the major factors responsible for failure were water induced hydrolysis and breakdown of the actual polymer by fungal activity. It was also shown that polyether type polyurethanes were more resistant to fungal attack than were the polyester types.

Kaplan et al. (1968) made a detailed review of the numerous studies carried out concerning the biodegradability of polyurethanes and discussed the fallacy of attempting to make meaningful evaluation of systems where the ingredients were unknown.

As a first step towards greater understanding of the biological susceptibility of chemically identified polyurethane systems, Darby and Kaplan (1968) investigated the extent of fungal growth on a series of laboratory synthesized polyurethanes and their components. In this study a relationship between chemical configuration and fungal susceptibility were examined. The results showed that the polyether-linked polyurethanes were, as a group, significantly less susceptible to fungal growth than the polyester-linked polyurethanes. They also suggested that the enzymatic

attack could occur only if there were sufficiently long unbranched carbon chains between the urethane linkages of the polymer, and that at least three adjacent methylene groups were required for appreciable attack to occur. The attack was found to be weaker if only two such groups were present. Examination of the diisocyanates showed that polyurethanes prepared with linear aliphatic diisocyanate were significantly more resistant to fungal attack than those prepared with the aromatic diisocyanates. This indicated that the proximity of the urethane linkages may interfere with enzyme accessibility to susceptible groups in the molecule.

Evans and Levisohn (1968) carried out studies on polyester type polyurethane printing rollers which were observed to become soft and deformed due to attack by fungi. Stemphylium sp. in particular was shown to be very aggressive. It made tunnels deep into the polyurethane within two days of incubation. Addition of anti-hydrolysis agents prevented penetration by Stemphylium sp. and autoclaved samples were attacked more readily than the unautoclaved ones. Both these observations led to the conclusion that the deterioration of the polyurethane printing rollers was due to the hydrolysis of the polymer.

Hedrick and Crum (1968) reported that the polyester type polyurethane foam used as a baffling material in aircraft fuel tanks was affected by the action of the jet fuel microbial isolates, Cladosporium resinae and Pseudomonas aeruginosa. It was evidenced by the production of extensive fungal matting in the rectangular matrix of the foam structure by

the fungal isolate, with fermentation and changes in the tensile strength of the physical structure of the foam, and an increased bacterial cell count in the presence of the foam and an increased oxygen uptake by the bacterial isolate.

Polyurethane systems have been reported to be attacked by marine organisms. Jones and Le Campion-Alsumard (1970) found that certain marine ascomycetes (Corollospora maritima, Haligena unicaudata, Lulworthia purpurea) and a fungi imperfecti (Zalerion maritima) caused degradation of polyurethane covered panels submerged for three years and eight months in the sea. The polyurethane panels were also observed to be susceptible to a bacterium which produced a salmon-pink colouration on the polyurethane.

In 1970, Dixit et al. reported that fungi caused severe deterioration of polyester type polyurethane foams used widely as a cushioning material and also as a packing material for costly instruments and equipment. Aspergillus niger and Paecilomyces sp. isolated from a degraded foam sample, had caused loss of cushioning property and elasticity of a fresh polyurethane foam when incubated at 30°C for twelve weeks. This foam sample was easily broken into small pieces on pressing, stretching and folding.

Awao et al. (1971) tested two commercial polyurethane rubbers (i. Urethane rubber E-4080: A product of Nippon Elastran Co., Tokyo. The chemical structure was not disclosed but it was probably a polyester type; ii. Urethane

rubber EVW-1018: A product of Hokusin Kagaku Co., Tokyo, chemical structure not disclosed.) for microbial resistance according to the "fungus resistance test" of the Japanese Industrial Standards. They observed that both urethane rubbers were radically attacked by Cladosporium herbarum, Penicillium citrinum, Chaetomium globosum and Aspergillus niger in liquid and on solid media after stationary incubation at 25°C for six months. They also noticed that it took about ten days for fungal mycelium to develop on the surface of the urethanes and also that the fungi grew better on the rough surfaces of the urethanes which had been cut by a knife, than on the smooth surfaces. The penetration of fungal mycelia into urethane rubbers too were observed microscopically after staining the microtomed sections of deteriorated urethanes with Cotton Blue.

Hole (1972) in a review briefly described some previously unpublished results on the biodeterioration of a polyurethane elastomer used in the shoe industry. Some strains of bacteria isolated from worn out shoes had completely embrittled this polyester polyurethane elastomer in four days at 30°C and 100% relative humidity. The addition of antimicrobial compounds to the elastomer retarded this effect although sweat-moisture absorption created a problem of gradual leaching away of the protective agent. Some polyurethanes known to have reasonably good hydrolysis resistance too had been embrittled in a few days by these bacteria.

Hole and Abbott (1971) (in Hole, 1972) showed that microorganisms can play an indirect role in the chemical deterioration process of polyurethanes and affect their rates. They suggested biological conversion of urea to ammonia could be detrimental to polyurethanes.

Decomposition of polyurethanes in a garbage landfill leakage water and by soil microorganisms was studied by Filip (1978). He measured the decomposition of resilient and hard polyurethane foams gravimetrically and using infrared spectrometry and found this decomposition to be more complete in polyurethanes based on polyesters and only very small in polyurethanes based on polyethers. His interpretation of the IR spectra led to the following sequence for the microbial decomposition of polyurethane:-

degradation of the remaining free isocyanate groups,

splitting of the urea and amide groups,

breaking off the urethane groups,

cleavage of the rings of the isocyanuric acid units.

Filip (1979) continued his study on the decomposition of polyurethanes using pure fungal cultures. Aspergillus niger and Cladosporium herbarum grew slowly in shake cultures with polyurethane foam as the sole nutrient source. Simultaneously, the isocyanate almost completely disappeared

from the polyurethane as determined with IR spectrometry. Otherwise, there were no structural changes found in the polyurethane, though both fungi penetrated into the resilient foam tested.

Griffin (1980) studied the tunneling of 'Daltrol', a cast polyester polyurethane elastomer, by Ulocladium chartarum (described by Evans and Levisohn, 1968, as Stemphylium sp.) and observed that no mechanical forces were at work in hyphal penetration. This penetration proceeded ahead of the growing hyphal tip and suggested that the tunneling process was primarily enzymatic.

Martens and Domsch (1981) studied the possibilities of the liberation of toxic aromatic amines from polyurethane foams under the influence of microorganisms, under different conditions of disposal. From the results they assumed that the polyether type polyurethanes were resistant to microbial as well as chemical attack under all practical conditions of disposal. Polyester type polyurethanes were susceptible to chemical hydrolysis favoured by extreme environmental conditions such as high temperature and/or low or high pH values. Under these circumstances they postulated that an accumulation of aromatic amines can occur if their further microbial degradation is impeded by the lack of suitable conditions for growth of microorganisms.

1.5 Objectives of the research programme

Although polyurethane polymers show outstanding

mechanical properties, good resistance to oxygen, ozone, ultraviolet, gamma radiation and aliphatic solvents and moderate resistance to dry and moist heat, hydrolysing media and oxygenated solvents, their resistance to micro-organisms, especially with polyester type polyurethanes, poses a serious problem. Even the great versatility of polyurethane chemistry has not been able to find a direct solution to this problem because certain polyester polyurethanes are essential for imparting certain characters which cannot be economically replaced by any other formulation. The use of biocides has not been the complete solution because of their incompatibilities with the particular polymer formulation.

From the literature it is clear that most of the work on biodeterioration of polyurethanes is empirical and no detailed study has been done to understand the mechanism of polyurethane deterioration and the importance of the deteriorating organism to achieve it.

Therefore, in this study, an attempt was made to understand the mechanism of polyurethane deterioration by understanding the mechanisms by which fungi deteriorate polyurethanes and also the conditions which encourage this to take place. This was achieved by isolating and selecting potential polyurethane deteriorating fungi and by establishing the contribution of the exoenzymes produced by them in the breakdown process of polyurethanes and the physical, mechanical and chemical changes which accompany this.

CHAPTER TWO

ISOLATION AND SELECTION OF TEST FUNGI

ISOLATION AND SELECTION OF TEST FUNGI

A number of fungi (Table 2.1) isolated from degraded polyurethane samples in natural environments were reported by Evans and Levisohn (1968), Hedrick and Crum (1968), Jones et al. (1970), Dixit et al. (1971), and Agarwal et al. (1971). When this list is compared with a list of standard test fungi (Table 2.2) used for evaluation of the resistance of plastics to microorganisms, it is apparent that there are other fungi which appear to have the specific ability to attack polyurethanes and which deserve investigation. Therefore, as the first step of this study, an attempt was made to isolate fungi from polyurethane samples undergoing biological attack which could be implicated as potential biodeteriogens of polyurethanes.

Polyurethanes undergoing biological attack were obtained from two sources. The first was a polyester type polyurethane product, which was probably undergoing microbial deterioration when in adverse storage conditions, and the other was a polyester type polyurethane poromeric material buried in a soil bed in the laboratory.

Isolation of a fungal species using this method does not simply imply that it is a potential deteriogen of polyurethanes. Fungal ecology with regards to colonization of polymeric materials poses many problems in itself (Hueck, 1974). For example, a fungus which is observed growing on a polymeric material may simply be existing

TABLE 2.1

FUNGI ISOLATED FROM DEGRADED POLYURETHANE MATERIALS

FUNGUS	SOURCE OF ISOLATION	REFERENCE
Stemphylium sp. (*Ulocladium chartarum*) *Penicillium citrinum* *Aspergillus niger* *Phoma sp.* *Fusarium sp.* *Cephalosporium sp.*	polyurethane printing rollers	Evans and Levisohn (1968)
Botryotrichum sp. *Mesobotrys sp.* *Pullularia sp.* *Scopulariopsis sp.* *Tetracoccosporium sp.*	aqueous leachates of printing rollers	
Haligena unicordata *Lulworthea purpurea* *Corollospora maritima* *Zalerion maritima*	polyurethane pieces submerged in sea	Jones et al. (1970)
Cladosporium resinae	baffling material	Hedrick et al. (1968)
Aspergillus niger *Paecilomyces sp.*	cushioning foam	Dixit et al. (1971)
Rhizopus sp. *Aspergillus flavus*	cushioning foam	Agarwal et al. (1971)

TABLE 2.2

FUNGI USED FOR STANDARD TEST PROCEDURES IN DETERMINING THE RESISTANCE OF PLASTICS TO MOULD ATTACK

FUNGUS	TEST METHOD
Aspergillus flavus	ASTM
Aspergillus glaucus	JIS
Aspergillus niger	ASTM, JIS, ISO
Aspergillus versicolor	ASTM
Chaetomium globosum	ASTM, JIS, ISO
Cladosporium herbarum	JIS
Paecilomyces varioti	ISO
Penicillium citrinum	JIS
Penicillium funiculosum	ASTM, ISO
Pullularia pullulans	ASTM
Trichoderma viride	ASTM, ISO

ASTM - American Society of Testing Materials

JIS - Japanese Industrial Standards

ISO - International Organisation of Standardisation

passively on it, not causing any deterioration, or alternatively, it may be actively deteriorating the material and utilizing it as a nutrient source. In addition to these problems there is also the possibility of fungi producing enzymes and other metabolites for some other function which in turn degrade the material.

Therefore, to select most probable potential biodeteriogenic fungi of polyurethanes, isolated fungi were subjected to two more experiments. In the first experiment the ability of isolated fungi to colonize the polyurethane material in the presence of a balanced organic nutrient source was studied. In the other experiment their ability to utilize the polyurethane as the sole source of nutrient for growth was studied. Results of these experiments gave some idea to select the potential biodeteriogens of polyurethanes from the others.

2.1 Isolation of fungi

The polyurethane materials used for fungal isolations were a polyester type polyurethane hard elastomeric commercial product suspected of having failed due to microbial attack as a result of adverse and prolonged storage; and a poromeric, polyester polyurethane material, primarily designed for construction of shoe uppers, which was buried in soil beds in the laboratory to initiate degradation by soil fungi.

Preparation of hard elastomer for isolation of fungi was done by cutting into small pieces (0.5cm x 0.5cm) aseptically and by subjecting to the treatments shown in figure 2.1.

The preparation of poromeric material for isolation of fungi was more complicated. First soil samples for soil burial experiment were collected from the top 10cm of relatively undisturbed loamy soil from the east end of St. Peter's College grounds, Saltley, Birmingham, which had a pH of 5.6. It was sieved through 1cm mesh to remove large particles and the soil beds (10cm deep) were prepared in HDPE boxes (90cm x 45cm x 30cm) covering them with polythene sheets to maintain a moisture content between twentyfive percent and thirty percent. Pieces (2cm x 0.5cm x 0.1cm) of poromeric material were buried vertically in soil beds about 3cm below from the surface and temperature was maintained at 25 $\pm$ 2°C. Eight pieces each were removed after 1, 2 and 4 weeks. Each piece was cut crossways into four aseptically. Half the number of pieces were kept unwashed whilst the remainder were washed in six changes of sterile distilled water with vigorously shaking in a mechanical shaker, for thirty minutes.

These poromeric pieces and the prepared hard elastomer pieces were plated on the following solid agar media in quadruplicate and in duplicate respectively. The media used were malt extract agar, Czapek-Dox agar, cellulose agar and malt extract agar with aureomycin (60mg/1l medium) to prevent bacterial contamination (Appendices 1a, 1b, 1c and

FIGURE 2.1

DIFFERENT TREATMENTS GIVEN TO DEGRADED POLYURETHANE PIECES BEFORE PLATING ON SOLID MEDIA

Degraded polyurethane pieces

(A)	(B)	(C)
Washed in a washing up solution for 15 minutes in a mechanical shaker and then with sterile distilled water.	Washed in Tween 80 (0.1%) for 15 minutes in a mechanical shaker.	No treatment
A1. Surface sterilisation with 70% industrial methylated spirits.	B1. Same as A1.	C1. Same as A1.
A2. Surface sterilisation with 0.1% H_2O_2 solution.	B2. Same as A2.	C2. Same as A2.
A3. Washed in sterile distilled water (5 times)	B3. Same as A3.	C3. Same as A3.

N.B. All pieces were surface dried between sterile filter papers (sterilized by autoclaving and drying in a laminar flow cabinet with microbiologically filtered air) before plating on solid media.

1d respectively). The plates were incubated at 25 ± 1°C and observations were made at regular intervals. Fungi were isolated from the mycelial fragments that grew out from the material, using a sterile pair of forceps. These isolated fungi were subcultured several times to obtain pure cultures and were maintained in malt extract agar slants.

Nineteen fungi (Table 2.3) were isolated from initial screening experiments. Of these, eleven were isolated from degraded polyurethane hard elastomer and eight from the poromeric polyurethane pieces buried in soil. Most of the fungi isolated were identified up to the species level and the remainder up to genus level, but, a specific code name was given to each fungus to indicate its source of isolation and thus avoid overlapping.

2.2 Selection of test fungi

Test fungi were selected after looking into capabilities of each fungus isolated and some known potential biodeteriogenic fungi obtained from pure culture collection of the University of Aston in Birmingham (Table 2.3), to colonize and also cause some sort of a deterioration of test polyurethane pieces in the presence and absence of another organic nutrient source.

To achieve this, pieces (1cm x 1cm) of poromeric polyurethane sheets were surface-sterilized with formalin vapour for three hours to prevent contamination of pure cultures used in these experiments. These were then washed

TABLE 2.3

FUNGI ISOLATED AND COLLECTED FOR SCREENING EXPERIMENTS

a. Fungi isolated from degraded polyurethane material

CODE	POSSIBLE IDENTIFICATION
G1	*Aspergillus flavus* Link
G2	*Aspergillus fumigatus* Fresenius
G3	*Aspergillus fumigatus*
G4	*Chaetomium globosum* Kunze
G5	*Mycelia sterilia*
G6	*Nigrospora spherica* (Saccardo) Mason
G7	*Paecilomyces varioti* Bainier
G8	*Penicillium notatum* Westling
G9	*Penicillium sp.* 1
G10	*Penicillium sp.* 2
G11	*Penicillium sp.* 3

b. Fungi isolated from poromeric pieces buried in soil

S12	*Fusarium oxysporum* Schlechtendahl
S13	*Fusarium sp.*
S14	*Gliocladium roseum* Bainier
S15	*Mucor sp.*
S16	*Penicillium chrysogenum* Thom
S17	*Scopulariopsis brevicaulis* Bainier
S18	*Trichoderma* (koningi) Oudemans
S19	*Trichoderma viride* Persoon

c. Fungi obtained from the University of Aston culture collection

L20	*Aspergillus fumigatus* Fresenius
L21	*Aspergillus niger* van Tieghem
L22	*Cladosporium herbarum* Link
L23	*Paecilomyces varioti* Bainier
L24	*Penicillium citrinum* Thom
L25	*Rhizopus rhizopodiformis*
L26	*Trichoderma viride* Persoon

thoroughly in sterile distilled water and were placed in petri plates containing a balanced organic nutrient source (malt extract agar) and a non-organic nutrient source [FA No.5 mineral salts agar (Berk et al., 1957) with 0.03% yeast extract]. Both types of petri plates were inoculated with unwashed spore or mycelial suspensions of fungi (Table 2.4) (N.B. in a few fungi where there was no or little sporulation, fungal mats were scraped and used as the inoculum). The plates were sealed in polythene bags to avoid desiccation and were incubated for four weeks at 25 ± 1°C. The degree of fungal colonization was measured according to the following ratings (ISO R 846, 1968):-

-1 zone of inhibition present

0 no fungal growth was apparent even under microscope

1 fungal growth was hardly visible to naked eye but quite apparent under microscope

2 slight visible growth covering less than 25% of surface

3 medium growth covering 25% - 50% of test surface

4 considerable growth covering 50% or more of surface

5 heavy growth covering entire surface.

Each fungus was tested in quadruplicate and each result (Table 2.4) was given as an average of four values.

As this experiment was done to study two different parameters, namely: a) the degree of colonization on malt extract agar to determine the degree of fungitoxic effects of the polyurethane and b) the capabilities of fungi to utilize the polyurethane as the sole source of nutrients, the results were assessed according to two standards.

The results of the experiment on malt extract agar (MEA) were evaluated as the degree of fungitoxicity of the poromeric material according to ISO R 846 (1968) recommendation as shown in the following table:-

GROWTH RATING	EVALUATION OF TEST MATERIAL
-1	Strong fungitoxic effect due to diffusing substance
0	Strong fungitoxic effect
1	Not quite complete fungitoxic effect
2 - 5	Decrease effectiveness to complete absence of fungitoxic effect

Five fungi (Table 2.4) were completely inhibited by the fungitoxic effects of the material. Eleven fungi showed varying degrees of inhibitory effects (ratings 1, 2 and 3) and the remainder (ten fungi) showed no inhibitory

effects when allowed to grow on the test material.

Further to these observations, the effects of fungal colonization on the poromeric material were studied. Although Rhiozopus rhizopodiformis, Mucor sp. and Trichoderma spp. completely covered the surface of the material, they did not show any signs of visual breakdown (described below) or discolouration of the poromeric material. Chaetomium globosum grew well on the material and changed the colour of the poromeric polyurethane from white to yellow-brown. Aspergillus fumigatus stained the poromeric material yellow and showed good growth on the material. Of all the fungi screened, only Nigrospora spherica and Gliocladium roseum showed visual degradation of the poromeric material. Nigrospora spherica stained the material with blue and violet spots and spores gave a black appearance to the material. It also showed surface etching of the material under low power magnification. Gliocladium roseum did the most extensive visual damage to the poromeric polyurethane material. It grew well and caused extensive pitting on the test pieces. The crater-like pits had diameters varying from 0.1mm to 2mm (figure 2.2).

The results of the experiment on mineral salts agar + 0.03% yeast extract medium were evaluated as the level of nutrients the poromeric polyurethane pieces supplied for growth of fungi according to ISO R 846 (1968) recommendations as shown in the following table:-

FIGURE 2.2

Crater like cavities caused by G. roseum on poromeric polyurethane material when plated on MEA medium up to 4 weeks

GROWTH RATING	EVALUATION OF TEST MATERIAL
-1 or 0	Test material is not a nutritive medium for fungi.
1	Test material contains nutritive substance or contaminated to such a small degree that they permit slight growth.
2 - 5	Test material is not resistant to fungal attack and provides nutritive substances for fungi.

According to the observations made (Table 2.4) only Penicillium citrinum, Trichoderma spp, Gliocladium roseum, Chaetomium globosum and Aspergillus fumigatus were capable of showing at least some growth on the poromeric material. Penicillium citrinum showed the best growth out of all the fungi screened.

2.3 Discussion

The results obtained in the experiment where poromeric polyurethane material was used as the main organic source did not give enough evidence to make a positive conclusion regarding the capabilities of the screened fungi to utilize it as the sole nutrient source for their growth and development.

Gliocladium roseum, Nigrospora spherica, Chaetomium globosum and Aspergillus fumigatus could degrade or stain the test material only in the presence of an additional

TABLE 2.4

GROWTH RATINGS OF FUNGI ON MALT EXTRACT AGAR AND MINERAL SALTS AGAR + 0.03% YEAST EXTRACT

CODE OF FUNGUS	GROWTH RATINGS ON MALT EXTRACT AGAR	GROWTH RATINGS ON MINERAL SALTS AGAR + 0.03% YEAST EXTRACT
G1	3	1
G2	1	0
G3	4	2
G4	4	2
G5	0	0
G6	5	1
G7	0	0
G8	0	0
G9	2	1
G10	2	0
G11	1	0
S12	3	0
S13	2	0
S14	5	2
S15	5	0
S16	2	1
S17	5	0
S18	5	1
S19	5	1
L20	1	0
L21	0	0
L22	1	0
L23	0	0
L24	3	2
L25	5	1
L26	5	1

source of nutrients. This suggested that: a) good growth is necessary for a fungus to be deteriogenic, b) fungal enzymes produced for some other purpose may in turn attack the polymer or, c) the byproducts of the metabolic reactions of the growing fungi may have detrimental effects on the polyester polyurethane poromeric material.

Using the results of these experiments five fungi were selected for further research work. They were: Gliocladium roseum, Nigrospora spherica, Chaetomium globosum, Aspergillus fumigatus and Penicillium citrinum.

Aspergillus fumigatus, as it was not reported in the literature as a biodeteriogen of polyurethanes and also it showed quite extraordinary morphological characters, it was sent to the Commonwealth Mycological Institute with Gliocladium roseum, another new fungus in the field of biodeterioration of plastics, for confirmation. Both were confirmed as Aspergillus fumigatus IMI 260418 and Gliocladium roseum Bainier IMI 260419 respectively. Chaetomium globosum is well known as a standard test fungus for determining the resistance of plastics to mould attack (Table 2.2). Penicillium citrinum too is used as a test fungus (Table 2.2) and also reported to be present in degrading polyurethane printing rollers (Table 2.1). Although Nigrospora spherica was not reported as a biodeteriogen of polyurethanes, it has been reported (Demmer, 1968) to degrade polyesters and polyamides.

As these fungi belong to quite different taxonomical

groups and they show a wide range of reactions with polyurethanes tested, it will be quite interesting to study their behaviour and performance as biodeteriogens of polyurethanes in the following experiments.

CHAPTER THREE

CHARACTERIZATION OF TEST FUNGI

CHARACTERIZATION OF TEST FUNGI

Although five test fungal species were selected from the initial screening experiments to study one aspect, namely: the biodeterioration of polyurethanes, taxonomically they varied quite considerably. It was thus considered necessary to establish some physiological parameters for use in the subsequent investigations. The following parameters were studied: a) effects of temperature and pH on the rates of growth and, b) an indication of their modes of attack on polyurethanes i.e. whether the attack was purely mechanical or whether exoenzymes excreted by fungi were at least partly responsible for the damage.

3.1 Effects of temperature and pH on growth of test fungi

Temperature and pH of the growth medium are known to be two critical parameters which affect the growth of fungi. Their response to temperature is important enough to be used as a criterion in classification. Fungi which cannot grow and sporulate below 35°C are called 'mesophiles' and others which can grow and sporulate at 45°C or more are called 'thermophiles' (Cooney and Emerson, 1964). Fungi which show characters between these two are called 'thermotolerants'.

The pH of the growth medium or substrate also affects the fungal growth (Cochrane, 1958). Every fungus shows its

optimum growth at a particular pH range and accordingly fungi can be termed acidophilic, neutrophilic and basophilic fungi. Acidophilic fungi prefer acidic pHs (pH 1 - 6), neutrophilic around neutral pH (pH 7) and basophilic the basic pHs (pH 8 - 12). But this is not sharply defined and most fungi show different degrees of growth between pH 2 and pH 10. In this study, importance of the response of test fungi to pH is due to the following reason: Polyurethanes contain a large number of acidic carboxylic and basic amine groups and these contribute a major portion of the breakdown products when the material is degraded, affecting the pH of the medium or substrate. Therefore, the response to pH will determine to a great extent the biodeteriogenic effects of these test fungi.

The two standard methods used to measure growth of a fungus are the colony radial growths on solid media and the specific growths in submerged cultures. Trinci (1969) studied in detail the use of both these methods in determining the effects of temperature on growth of Aspergillus nidulans. He observed that the changes in specific growth rates of Aspergillus nidulans in submerged culture brought about by temperature changes were reflected in colony radial growths at least over most of the range tested. He concluded that colony radial growths would be a reliable parameter to use to determine the optimum temperature of growth of a mould, but that is not a meaningful parameter of growth when comparisons are made between species.

Allsopp (1973) used the colony radial growth method to study the effect of pH on growth of Trichoderma viride, but did not compare these results with the specific growth measurements in submerged cultures. Mills and Eggins (1970) used only the specific growth measurements in liquid cultures to study the effect of pH on growth of Aspergillus fumigatus at 40°C in a carboxylic acid medium.

3.1.1 Materials and methods

The procedure used to measure colony radial growths of the test fungi at different temperatures and pHs was as follows:-

Malt extract agar (MEA) plates (diameter 9cm), each containing 10 ml medium, were inoculated with plugs of mycelia (diameter 0.5cm), cut using a sterile cork borer (No. 2), from week old cultures of test fungi. The plugs were placed with the mycelium facing the agar medium. The plates were then sealed inside polythene bags to avoid desiccation during incubation. Growth was recorded by measuring the diameter of colonies in two directions at right angles and taking the mean of five replicates. The results were expressed as:

radial growth (cm) = (final growth diameter, cm) - (diameter of mycelial plug, cm)

In the experiment to study the effects of temperature

on growth, the plates were inoculated with Gliocladium roseum, Chaetomium globosum, Penicillium citrinum, Aspergillus fumigatus and Nigrospora spherica and were incubated at 20°, 25°, 30°, 35°, 40° and 45°C for 3 days and the growth diameters were measured.

In the determination of colony radial growths at different pHs, the pH value of the MEA medium was adjusted using the buffer solutions as follows: pH 3 to pH 6 - citrate-phosphate buffer; pH 7 to pH 8 - phosphate buffer; pH 9 - tris-HCl buffer (Gomori, G., 1955). The buffer solutions and media were prepared double strength, autoclaved separately and then mixed after cooling to about 45°C to avoid any hydrolysis. The plates were incubated at 30°C and the growth diameters of Chaetomium globosum were measured after 3 days and the remainder, Gliocladium roseum, Penicillium citrinum, Aspergillus fumigatus and Nigrospora spherica after 6 days.

In addition to colony radial growths at different pHs, specific growths in submerged cultures at different pHs were measured according to the following procedure:

100ml conical flasks, each containing 50ml of malt extract broth (appendix 1e) buffered at pHs 3, 4, 5, 6, 7, 8 and 9 using the earlier mentioned buffer systems, were inoculated with plugs of mycelia (diameter 0.5cm), cut using a sterile cork borer (No. 2). The inocula were taken from week old cultures of Gliocladium roseum, Chaetomium globosum,

Penicillium citrinum, Aspergillus fumigatus and Nigrospora spherica. The flasks were incubated static at 30°C for 7 days with a gentle shaking for one minute each day. After incubation, the mycelial mats were filtered through dried and weighed Whatman No. 1 filter papers. The filtered mycelium was washed several times with distilled water and dried in an oven at 55°C to a constant weight. Controls were set up without incubation to measure the dry weights of original agar plugs. The specific growths were given as dry weights of filtered mycelium and were calculated as:

$$\left[\begin{array}{l}\text{dry weight of}\\ \text{filtered mycelium (g)}\end{array}\right] = \left[\begin{array}{l}\text{final weight of}\\ \text{filtered mycelium (g)}\end{array}\right] - \left[\begin{array}{l}\text{weight of}\\ \text{the agar}\\ \text{plug (g)}\end{array}\right]$$

Each experiment was done in triplicate.

3.1.2 Results

Colony radial growth measurements of test fungi at different temperatures are shown in figure 3.1. Maximum growth diameters of Gliocladium roseum, Chaetomium globosum and Penicillium citrinum were attained at 30°C. Nigrospora spherica showed maximum growth between 25° and 30°C. The growth rates of all these fungi dropped sharply beyond 30°C and they ceased to grow after 40°C. Aspergillus fumigatus, on the other hand, showed a gradual increase in radial growth up to 35°C and then decreased quite rapidly, but some growth was observed even at 45°C, the maximum test temperature.

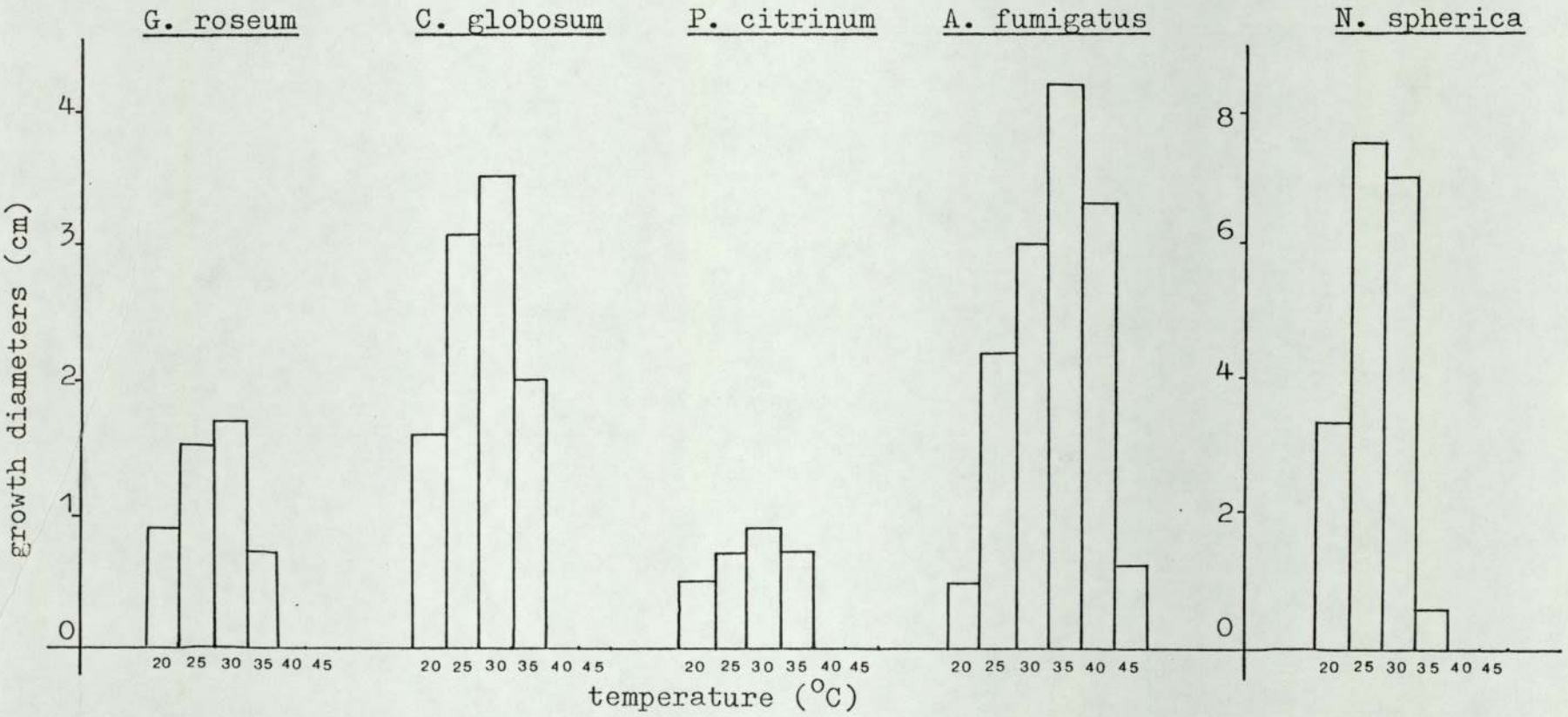


FIGURE 3.1

Radial growth diameters of test fungi at different temperatures after 3 days incubation

The growth measurements of test fungi at different pH values are shown in figure 3.2. No growth results for Nigrospora spherica were given because it ceased growth during the experiment and all attempts to reculture it failed. There was good correlation between radial growth measurements and specific growth measurements in submerged cultures. Gliocladium roseum and Chaetomium globosum showed a broad peak with a maximum at pH 7. Penicillium citrinum attained optimum growth at pH 6 and Aspergillus fumigatus at pH 4. All four fungi showed varying degrees of growth and changes in morphology throughout the pH range tested.

3.2 Studies on mode of attack of test fungi on polyurethanes

The possible ways of fungal deterioration of plastics are by active physical penetration of the material by the growing mycelium, or by chemical deterioration due to catalytic action of enzymes produced by the fungus on breakdown of polymer chains, or by penetration of the hyphae aided by enzyme action. Therefore every fungus capable of deteriorating polyurethanes may possess at least one of these modes of attack.

Past literature on biodeterioration of polyurethanes provide evidence for these modes of fungal attack. Evans and Levisohn (1968) showed that Stemphylium sp. actively penetrated polyurethane printing rollers causing deep tunnels; Aspergillus niger, Fusarium sp. and Cephalosporium sp. produced limited amounts of penetration just below the surface of the polyurethane; and Penicillium citrinum and

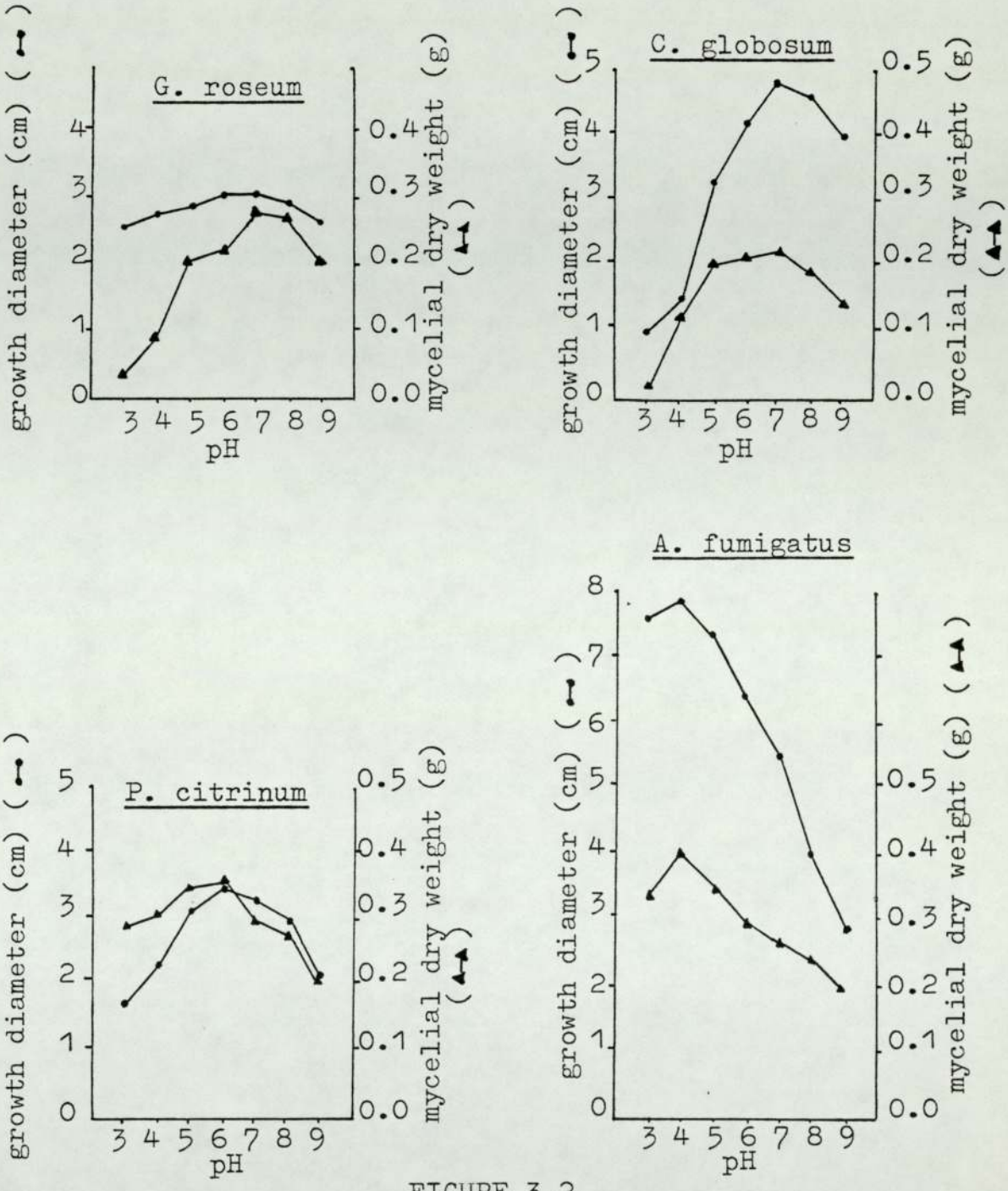


FIGURE 3.2

Study of the effects of pH of the medium on growth of test fungi using radial growth diameters and mycelial dry weights

Phoma sp. grew only on the surface of the polyurethane. Awao et al. (1971) showed penetration of mycelia of Cladosporium herbarum, Penicillium citrinum, Aspergillus niger and Chaetomium globosum into urethane rubbers by staining sections of the deteriorated polymer with cotton blue. Griffin (1980) revealed that tunneling of 'Daltrol', a polyester type polyurethane, by Ulocladium chartarum was due to penetration of growing mycelium aided by enzyme action.

In the present study, a method was devised to study whether the deterioration of polyurethanes by the test fungi were purely mechanical or whether exoenzymes too contributed significantly to this effect.

3.2.1 Materials and methods

Three polyester type polyurethane elastomers (yellow soft, red medium and red hard (appendix 2)) were used as the test materials.

Surface-sterilized test polyurethane pieces (1cm x 0.3cm x 0.05cm) were embedded in MEA medium and then covered with a sterile membrane filter (0.45 um, Gelman Science Inc., Michigan, U.S.A.) (figure 3.3). A 0.5 ml spore suspension of test fungi was then carefully spread over the surface of the plate. After incubation at 30°C for one month, the test pieces were removed very carefully to avoid any possible contamination from the cultures, by cutting away the bottom

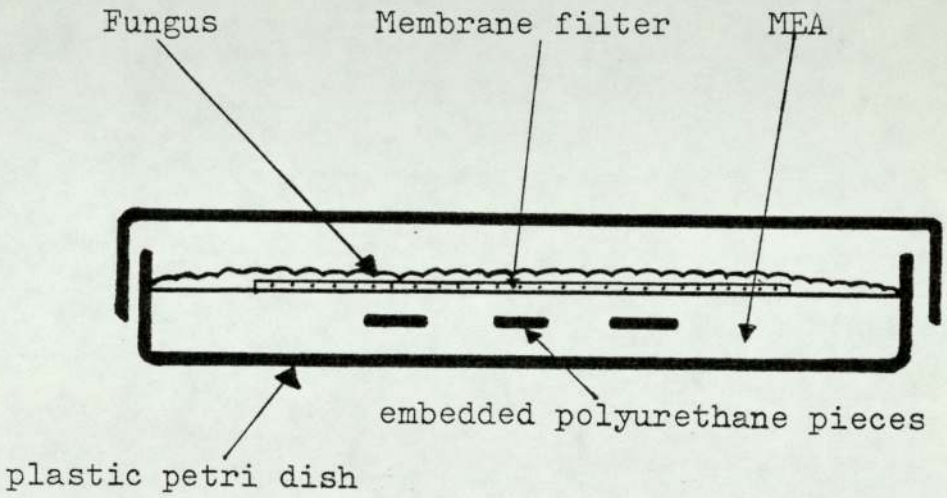


FIGURE 3.3

Method to determine whether exoenzymes of test fungi are responsible for deterioration of polyurethanes

of the plastic petri dish. They were then plated on to fresh MEA medium and incubated at 30°C for a week to see whether there was any growth from the test pieces. This check was carried out to ensure that no hyphae had made contact with the test piece by penetrating the membrane filter and the medium.

3.2.2 Results

All test pieces embedded and covered with membrane filters showed degradation to different degrees. Maximum degradation was observed with Penicillium citrinum, where the pieces were almost completely embrittled. The attack was to a lesser degree with Gliocladium roseum, Aspergillus fumigatus and least with Chaetomium globosum. The surface facing the fungus was mainly degraded, and under the microscope erosion troughs and cavities were observed. The replated pieces did not give rise to any fungal mycelia on fresh MEA medium after seven days incubation at 30°C.

3.3 Discussion

Growth studies at different temperatures showed that Gliocladium roseum, Chaetomium globosum, Penicillium citrinum and Nigrospora spherica were typical mesophilic fungi with optimum temperatures for growth between 25°C and 30°C. Aspergillus fumigatus attained maximum growth at 35°C and continued growth even at 45°C exhibiting thermotolerant features. From these results, 30°C was selected as the

standard test temperature to be used in the later experiments.

In the determination of the effects of pH on the growth of the test fungi, colony radial growths on solid media, and specific growths in submerged cultures were compared. Results from both methods showed good correlation, but, with slow-spreading fungi like Penicillium citrinum and Gliocladium roseum, the effects were more pronounced in specific growth measurements in submerged cultures. Each fungus responded in its own way to changes in pH. The observations made with Gliocladium roseum were similar to the results of Isaac (1954) and Burrows (1980). Chaetomium globosum too has been reported to show good growths in neutral and alkaline pHs (Burrows, 1980). Mills and Eggins (1970) reported that Aspergillus fumigatus grew better at alkaline pH values, but, the observations made with the strain of Aspergillus fumigatus used in this study indicated that it prefers acidic pHs. This is further supported by observations made on straw (Burrows, 1980). Penicillium citrinum too preferred slightly acidic pHs as observed in the present work.

The experiment to study the presence and effects of exoenzymes has indicated that the degradation of polyurethanes by all test fungi was at least in part due to exoenzymes produced by the fungus. The negative results with the replated pieces showed that the degradation was not due to penetrated hyphae. These observations indicate that all test fungi use a chemical mode of attack to break down polyurethanes and thus it is not only active penetration.

Added to these, it showed that each fungus produced enzymes of different effectiveness or concentrations. These effects will be studied further in Chapter Four.

CHAPTER FOUR

ENZYME ACTIVITIES OF TEST FUNGI

ENZYME ACTIVITIES OF TEST FUNGI

The importance of enzymes produced by fungi in biodeterioration of polyurethanes has been suggested in the literature by many workers.

Evans and Levisohn (1968) suggested that breakdown of polyester polyurethane printing rollers by fungi was due to hydrolysis of ester bonds by esterase produced by fungi. Hole (1972) reported that microorganisms could deteriorate poromeric polyurethanes by hydrolysis of ester bonds probably by esterases and also by hydrolysis of urea in skin exudates by ureases to produce ammonia, which could be detrimental to polyurethanes. Filip (1978) suggested that ureases, esterases and histidases produced by fungi could be detrimental to polyurethanes. Griffin (1980) showed the enzymic dissolution of a polyester polyurethane elastomer by Ulocladium chartarum.

Earlier experiments in the present study (Chapter 3) indicated that all the fungi deteriorated polyurethanes chemically using the enzymes they produced. To achieve this, they should possess enzyme systems which could attack the ester, urethane, amide, urea, ether and biuret bonds present in the polymer. The known enzymes which are capable of this are esterases, proteases and ureases. Therefore, an understanding of these enzyme activities of test fungi will be a very important step in determining their ability to chemically degrade polyurethanes.

Enzyme production by fungi is usually assayed (Fodor and Chari, 1948; Soru et al., 1965; Mills and Eggins, 1974; Lazar, 1975) by grinding the mycelium in a buffer and taking the extract and also by taking the culture filtrates. These methods can give misleading estimates of actual enzyme activities (Rautela and Cowling, 1966) because: a) measurements usually are made after a single arbitrary selected harvest time, that may not be optimal for synthesis of enzymes by all organisms, b) components of enzyme systems can be denatured during isolation, c) portions of enzyme system may be excluded from the analysis because they are bound to the mycelium of the fungus thus do not appear in the filtrate and, d) products of enzyme action may accumulate and prevent the hydrolytic action going to completion.

The use of solid agar media for detection of enzyme production by fungi has the advantage of measuring the activity of growing cultures rather than culture filtrates, thus involving less losses due to product inhibition, and binding or denaturation of enzymes. Earlier experiments on solid media (Hankin and Anagnostakis, 1975) involved measurement of diameters of clear zones (amylase, protease and esterase activity determinations) by using living cultures. In tests with living cultures, the extent of clearing is often obscured because it must be viewed through the mycelium of the organism itself. Also irregular patterns of clearing often result from uneven growth so that accurate measurements of diameter are difficult to make. Using this method the only way to make the assays semiquantitative is by

measuring the diameter of the zones formed in agar and relating this zone to the diameter of the fungal colony. In this manner, the enzymatic activity of variants of same species may be determined. However, such relationship may not be valid when different species are compared with each other.

Rautela and Cowling (1966) put forward a technique to overcome these problems in the determination of cellulase activity. They suggested the measurement of depth of clearance beneath the growing cultures. This method had the following advantages when compared with conventional assay procedures. They are: a) results of fungi of different species can be compared with each other, b) this method measures activity of growing cultures, so that less risk is involved of losses due to product inhibition, binding or denaturing of enzymes, c) repeated measurements can be made on the same experimental set up, thus minimizing influence of variation among set ups, d) it requires less working time and simple equipment, so that it is convenient for large scale screening tests. Therefore this method was modified to suit determinations of amylase, protease, esterase and urease activities in the following set of experiments.

4.1 Selection of a suitable basal medium

It is sometimes necessary that a medium used for determining enzyme activities of a fungus should provide sufficient growth factors, other than the test substrate to allow reasonable initial growth of the fungus. A mineral

salts medium (appendix 1f) was used by Berk et al. (1957) in determination of esterase activities of fungi. Rautela and Cowling (1966) used micro-quantities of thiamine HCl, adenine, adenosine and also 0.05% yeast extract in addition to mineral salts in the basal medium for cellulase activity measurements. Hankin and Anagnostakis (1975) used Difco nutrient agar and Difco plate count agar as the basal media to determine amylase, protease and urease activities. In the present experiment three different media were tested to select a suitable basal medium for subsequent use.

Specific growths in liquid cultures were used as the criterion to select the suitable medium. The media tested were:-

1. FA No. 5 mineral salts medium (appendix 1f) /NSM/

2. 0.5% mycological peptone + 0.3% lab lemco powder medium /PLM/

3. NSM + PLM medium.

Controls used were:-

4. Only water

5. NSM + 3% malt extract medium.

6. PLM + 3% malt extract medium.

7. NSM + PLM + 3% malt extract medium.

The same method (Chapter 3.1.1) used to study the specific growths of test fungi at different pHs was used in this experiment. Dry mycelial weights were determined after incubation for two weeks.

The results are presented in figure 4.1. There was very poor or no growth in NSM medium. In the other two test media, namely: PLM and NSM + PLM, there was moderate growth relative to the growth in control media with 3% malt extract and the control with distilled water, thus satisfying conditions for a suitable basal medium. But, the PLM medium had the added advantages of being simple and ease of adjustment of pH. Therefore it was selected as the basal medium for future enzyme studies.

4.2 Enzyme activity determinations

Amylase, protease, esterase and urease enzyme production of test fungi were studied on solid media. The method used was a modification of Rautela and Cowling's (1966) test tube method, where depth of clear zone/colour change that developed beneath the growing cultures provided a visual measure of enzyme activities on a continuous, cumulative basis.

The procedure for enzyme activity determinations was

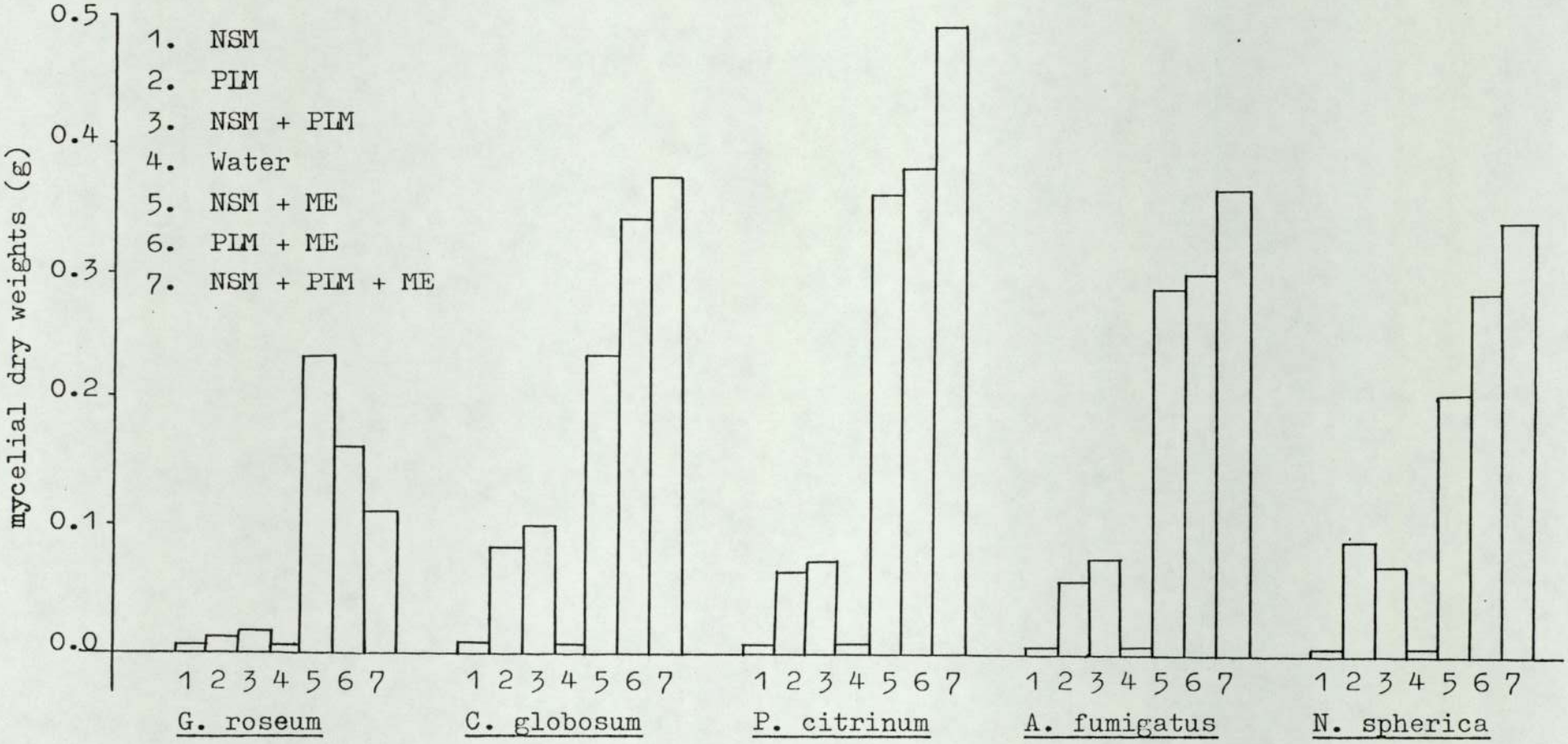


FIGURE 4.1

Growth measurements of test fungi in different media using the mycelial dry weights

as follows:-

Test tubes (1.2cm diameter x 10cm) with suitable oxoid caps were sterilized in a hot air oven at 160°C overnight. 5ml each of sterile, cooled (about 45°C) test media were added to test tubes, using a sterile automatic pipette syringe. When cooled, they were inoculated with plugs of mycelia (diameter 0.3cm), cut using a sterile cork borer (No. 1), from week old cultures of Gliocladium roseum, Chaetomium globosum, Penicillium citrinum, Aspergillus fumigatus and Nigrospora spherica. The plugs were placed with the mycelium facing the agar medium. The tubes were incubated at 30°C before enzyme activities were measured. Controls were set without inoculation. Each experiment was done with five replicates.

The ability to degrade starch was used as the criterion for determination of ability to produce amylolytic enzymes. The medium used (appendix 1g) contained 0.2% soluble starch. After incubating for seven days the agar columns were carefully taken out from the tubes by passing water under the column using a hypodermic needle (15cm) and a syringe. The removed agar columns were dipped in an iodine solution (0.5% I_2/KI) for 15 minutes and the depth of clearance in an otherwise blue column was measured.

A medium that contained 0.4% gelatin as the protein substrate (appendix 1h) was used to detect production of protease enzymes. After incubation for seven days the agar

columns were carefully removed and were dipped in saturated $(NH_4)_2SO_4$ solution for two to four hours. The depth of clearance in an otherwise opaque agar column was measured.

Production of enzymes capable of hydrolysing ester bonds was tested using 0.5% of a polyester (ethylene glycol/ butane diol (50:50) adipate, molecular weight about 2000) medium (appendix 1i). The medium was blended under sterile conditions using a high speed mechanical stirrer and the tubes were poured just before the agar hardened, to avoid any separation of the emulsion. After seven days incubation the depth of clearance in an otherwise opaque column was determined.

Change of pH due to hydrolysis of urea was used as the criterion to detect production of ureases. The indicator used to observe the change of pH was bromothymol blue and the colour change was from yellow to blue. Selected basal medium could not be used in this experiment because the colour changes occurred even in the controls without urea although to a much lesser extent. Therefore a new medium with 1% malt extract and 0.5% urea was used (appendix 1j). The tubes were incubated for three days and the depth of colour change was measured. Additional controls without urea were set up , inoculated and incubated parallel to the test samples.

4.2.1 Results

The depth of clearance/colour change due to amylase,

protease, esterase and urease activities of Gliocladium roseum, Chaetomium globosum, Penicillium citrinum, Aspergillus fumigatus and Nigrospora spherica are presented in figure 4.2.

Penicillium citrinum and Gliocladium roseum showed good all round activity. Chaetomium globosum indicated good urease activity, moderate protease activity and low esterase activity. Nigrospora spherica showed low urease activity moderate esterase activity and high protease activity. Unlike others, Aspergillus fumigatus showed very little or no protease and urease activities, but it had very high esterase activity. All test fungi showed good amylase activities.

In esterase activity determinations, Penicillium citrinum and Chaetomium globosum completely cleared the opaque medium above the clearance margin. So the margin was very pronounced. The remainder of the fungi incompletely cleared the opaque medium above the clearance margin making it less pronounced.

A completely saturated ammonium sulphate medium was necessary to see the margins clearly in protease activity determinations. Precipitation of unhydrolysed gelatin is hastened if the saturated solution was stirred regularly.

In urease activity measurements, the margin of colour change was not very distinct. Although there was no definite

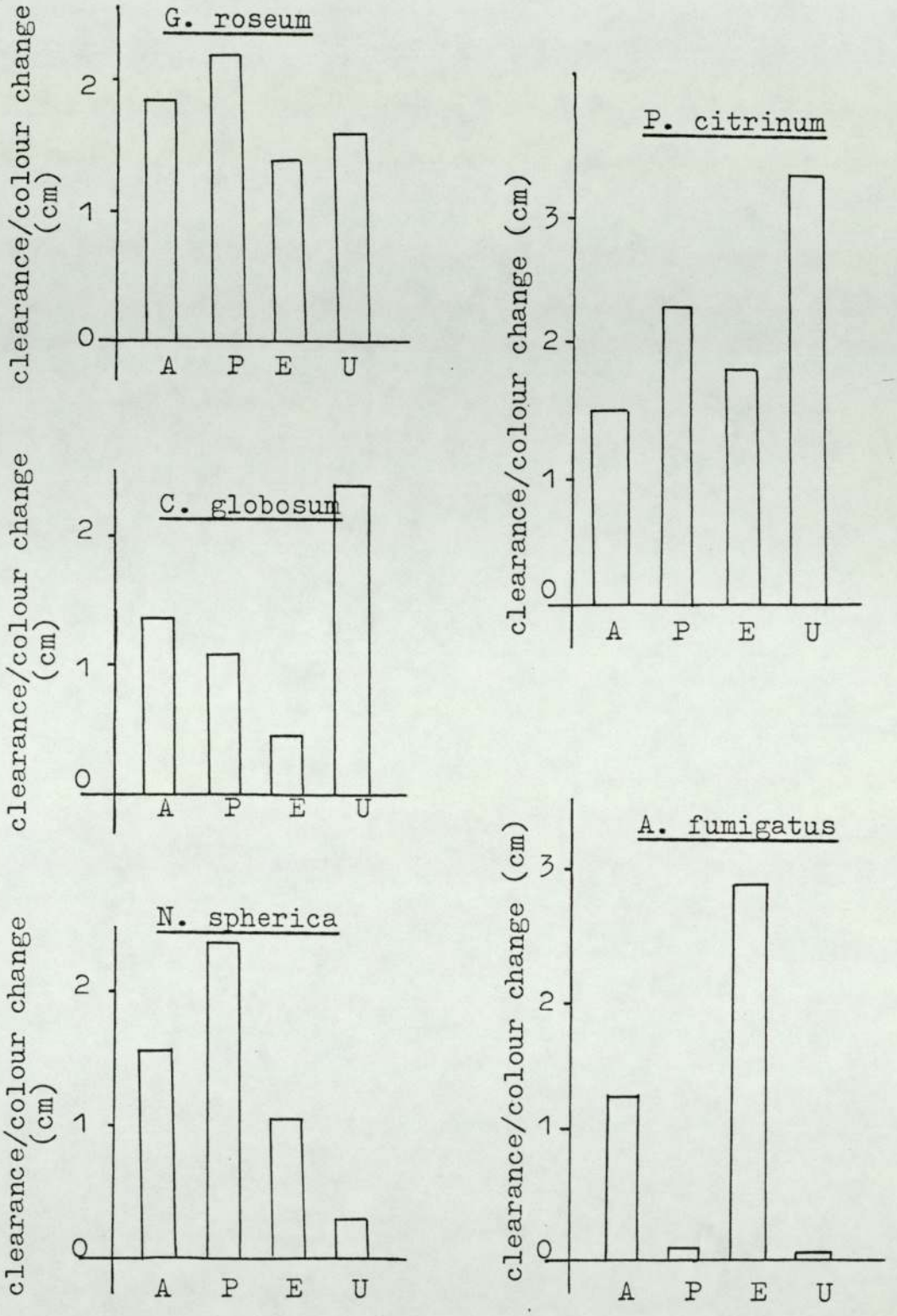


FIGURE 4.2

Enzyme activities of test fungi

A = amylase activity

P = protease activity

E = esterase activity

U = urease activity

The standard deviations between replicates were $\pm$ 0.1 cm

margin of colour change, it was easy to set an arbitrary margin to measure the depths of colour change and thus measure urease activity. Another important observation was that the intensity of blue colour varied considerably with different fungi. The maximum intensity was observed with Penicillium citrinum, then with Chaetomium globosum, Gliocladium roseum, Nigrospora spherica and Aspergillus fumigatus in decreasing order.

4.3 Effects of pH on enzyme activities

pH values of the test media used to determine amylase, protease, esterase and urease activities were adjusted using buffer solutions as follows:- pH 3 to pH 6 - citrate-phosphate buffer; pH 7 to pH 8 - phosphate buffer; pH 9 - tris-HCl buffer (Gomori, G., 1955).

The buffer solutions and media were prepared double strength, autoclaved separately, and then mixed after cooling to about 45°C to avoid any hydrolysis.

The test tube method of the measurement of depth of clearance (see section 4.2) was used to study the effects of pH on amylase, protease and esterase activities. The same media used earlier were buffered to obtain the necessary pHs. The tubes were inoculated with the test fungi and were incubated at 30°C for 7 days. Enzyme activities were measured as before, controls were set up without inoculation. Each experiment was done in triplicate.

The same could not be done with the urease activity determinations at different pH values because the criterion used to detect the enzyme activity was changes in pH values. Therefore a titrimetric method was used to determine effects of pH on urease activity of test fungi.

50ml each of sterile buffered medium (appendix 1k) in 100ml conical flasks at pHs 3, 4, 5, 6, 7, 8 and 9 were inoculated with plugs of mycelia (diameter 0.5cm) cut using a sterile cork borer (No. 2) from week old cultures of test fungi. They were incubated at 30°C for 10 days. Controls were set without inoculation. After incubation, the medium was filtered through Whatman No. 1 filter papers, 10ml of filtrate were titrated against 0.05N HCl or 0.05N NaOH to a common end point using bromothymol blue as the indicator. Urease activity was measured as the amount 0.050 N HCl used per 10ml medium and was calculated as:

$$A = B - C$$

where:

A is (urease activity) amount of 0.05 N HCl used/10ml medium

B is Amount of 0.050 N HCl used or (-) amount of 0.050 N NaOH used in test solution

C is Amount of 0.050 N HCl used or (-) amount of 0.050 N NaOH used in control

Each experiment was done in triplicate.

In these experiments to study the effects of pH on enzyme activities of fungi, only four test fungi were used. They were Gliocladium roseum, Chaetomium globosum, Penicillium citrinum and Aspergillus fumigatus. Nigrospora spherica suddenly ceased growth and all attempts to reculture it failed.

4.3.1 Results

The effect of pH on amylase activity of test fungi is shown in figure 4.3 as depths of clearance at different pH values. Amylase activity of Gliocladium roseum, Chaetomium globosum and Penicillium citrinum increased gradually from pH 3 to pH 8 and then decreased rapidly. But, Aspergillus fumigatus showed maximum amylase activity at pH 3 and then another peak at pH 6 and pH 7.

All fungi tested showed (figure 4.4) maximum protease activities at neutral pH (pH 7). The activities gradually increased from pH 3 to pH 7 and then gradually decreased to pH 9 except in Penicillium citrinum where there was a peak at pH 4. Protease activity of Aspergillus fumigatus remained very low at all pHs tested.

The effects of pH on esterase activities varied considerably among test fungi (figure 4.5). Penicillium citrinum showed maximum activity at pH 5, Aspergillus fumigatus between pH 5 and pH 6, Gliocladium roseum at pH 6 and Chaetomium globosum at pH 7. All fungi showed only one

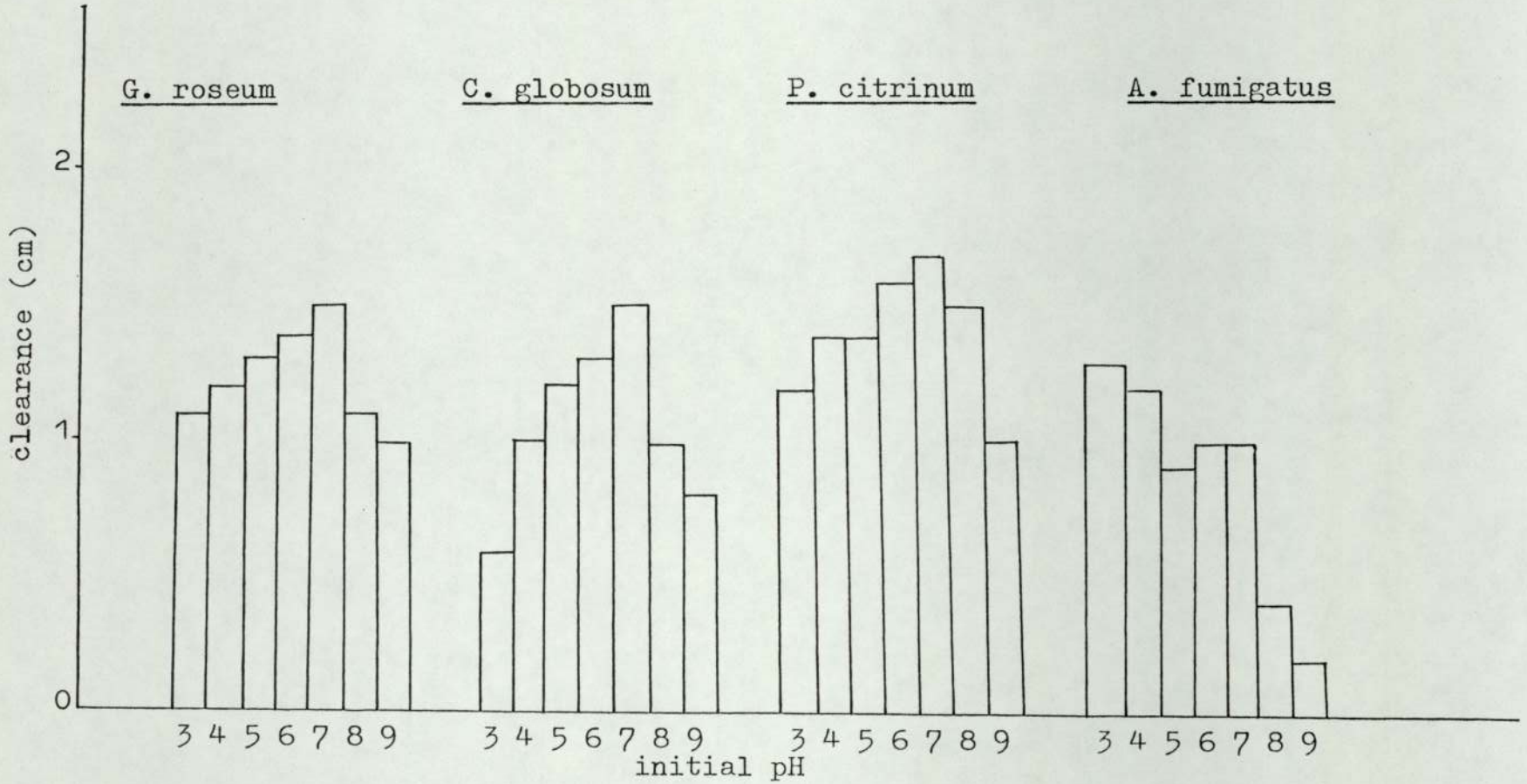


FIGURE 4.3

Amylase activities at different pH values

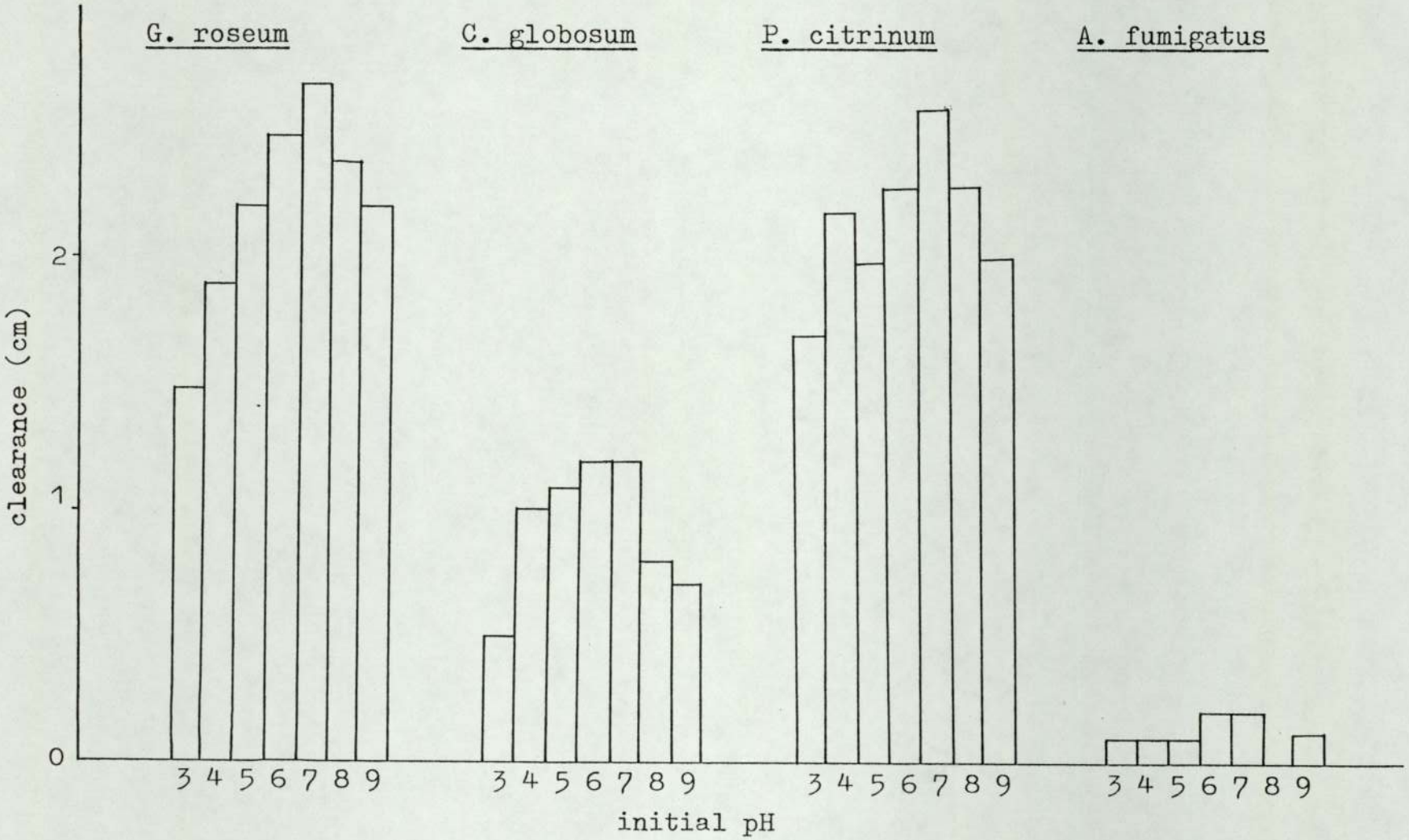


FIGURE 4.4

Protease activities at different pH values

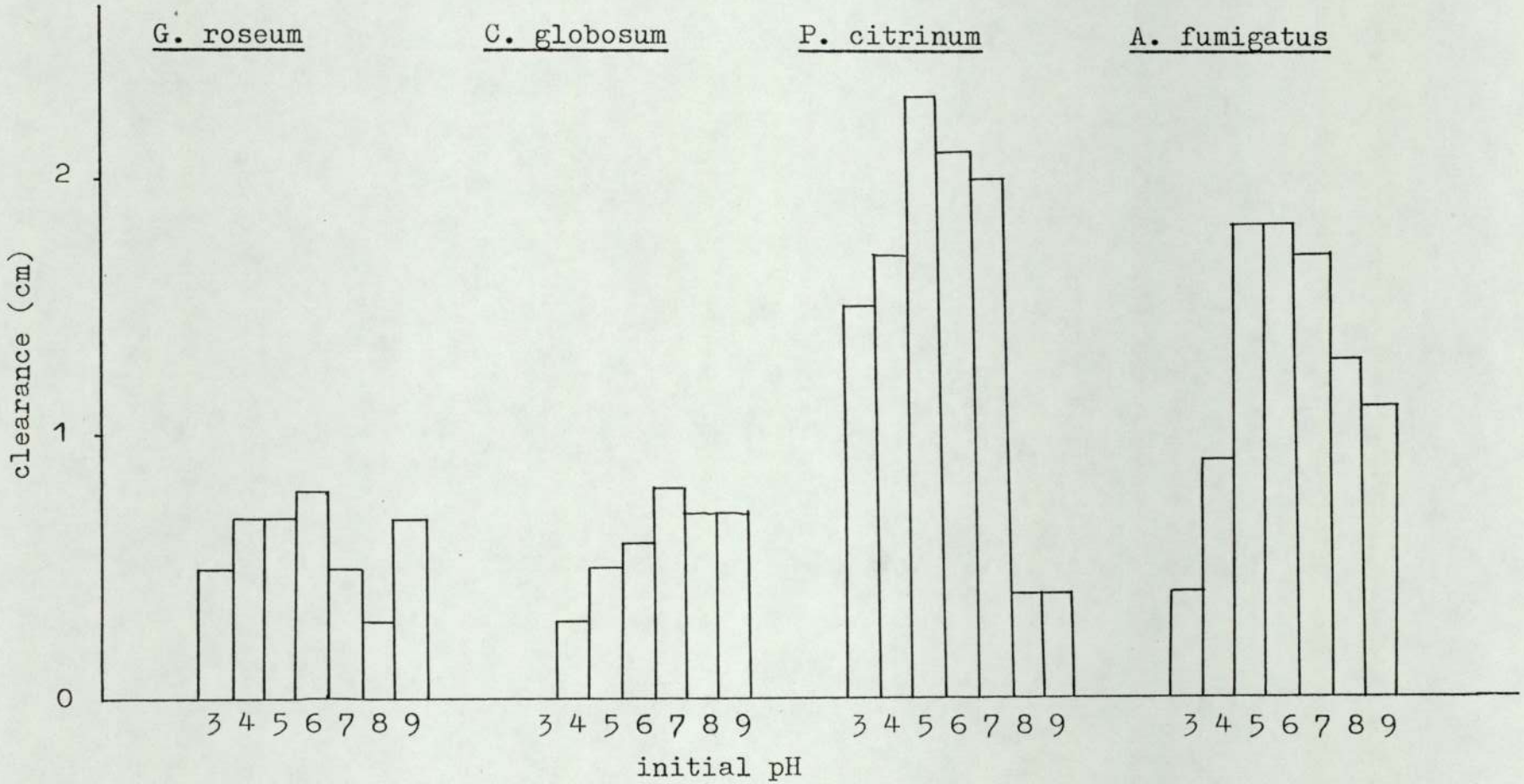


FIGURE 4.5

Esterase activities at different pH values

peak except Gliocladium roseum which had another peak at pH 9.

Urease activities of test fungi at different pH values are shown in figure 4.6. Higher activities were observed in acidic and neutral pHs. Penicillium citrinum showed maximum activity at pH 3 and the remainder at pH 4. Only Chaetomium globosum had an additional peak at pH 9. The smell of ammonia was observed in media with initial pHs from pH 3 to pH 8 with Penicillium citrinum, from pH 5 to pH 9 with Chaetomium globosum and from pH 4 to pH 7 with Gliocladium roseum. No smell of ammonia was observed at any pH value with Aspergillus fumigatus.

4.4 Effects of additional nutrients in the media on enzyme production.

Amylase, protease, esterase and urease activities of test fungi were compared up to 4 weeks on a limited nutrient, 0.5% peptone + 0.3% lab-lemco agar medium (PLA) (appendix 11) and on a balanced nutrient, malt extract agar medium (MEA).

Depth of clearance/colour change method was used with slight modifications. The test substrates, such as starch (0.2%), gelatin (0.4%), polyester (0.5%) and urea (0.5%) were added directly to PLA and MEA media. 15ml of medium was used in each test tube (1.6cm diameter x 20cm) which was then inoculated with plugs of mycelia (0.5cm diameter) cut using a cork borer (No. 2). The tubes were incubated

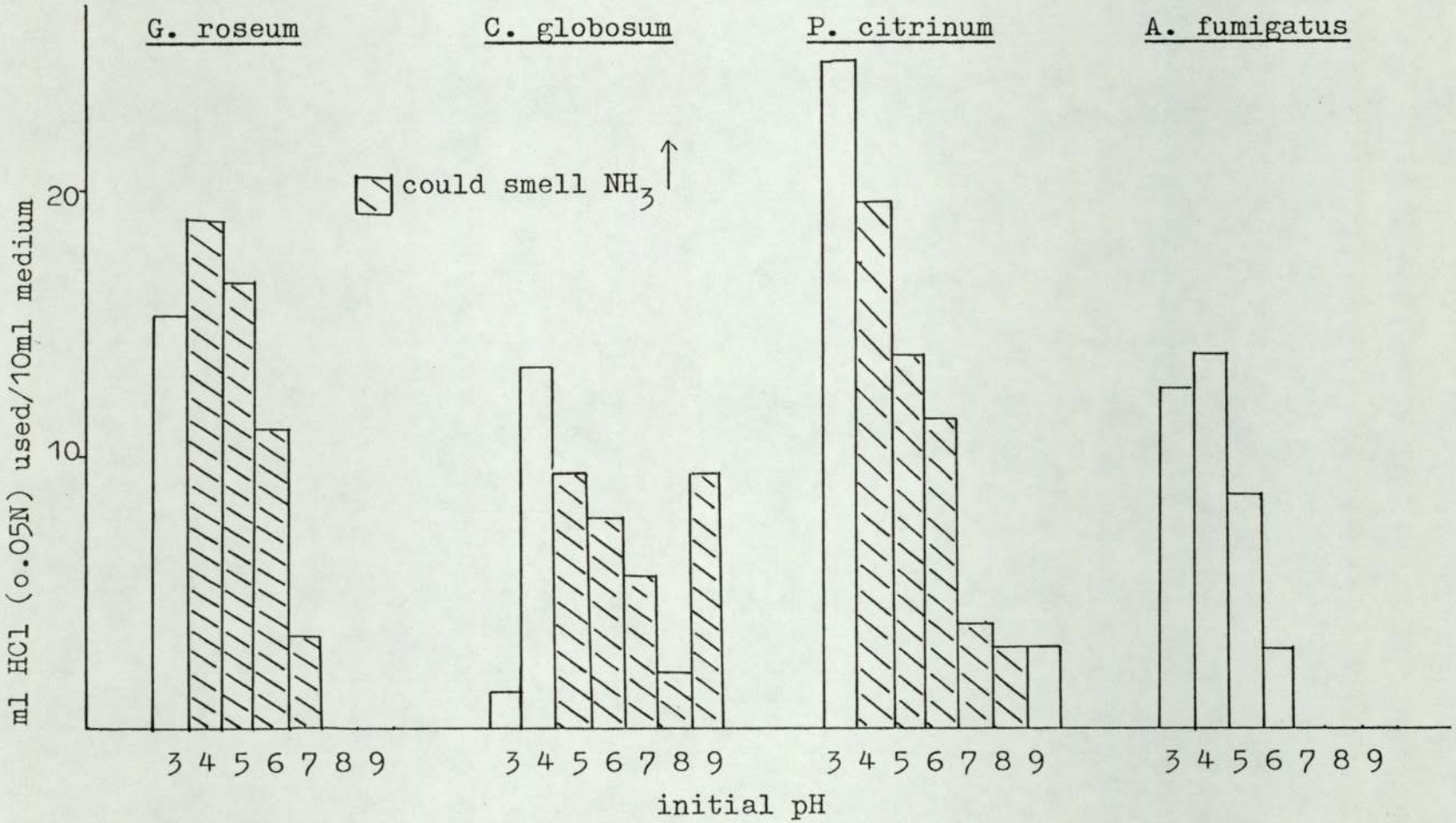


FIGURE 4.6

Urease activities at different pH values

at 30°C and depths of clearance/colour change were measured after 1, 2 and 4 weeks. Each experiment was done with six replicates.

4.4.1 Results

Figures 4.7 to 4.10 indicate the amylase, protease, esterase and urease activities respectively of test fungi up to 4 weeks on MEA and PLA media. In general, the enzyme activities on PLA medium were significantly higher than those on MEA medium.

The least pronounced difference of activity on different media was observed with amylase activity. Aspergillus fumigatus did not show any protease or urease activity on both media. The gap between protease activities on different media of Gliocladium roseum and Penicillium citrinum closed significantly with time, but it increased gradually with Chaetomium globosum. A problem encountered in the urease activity determinations was that the agar columns changed colour completely before the completion of the experiment.

Interesting observations were made on esterase activity measurements. Gliocladium roseum and Chaetomium globosum did not show any esterase activity on MEA even after 4 weeks. Penicillium citrinum too did not produce any esterases on MEA medium in the first week but started producing after two weeks. Very high esterase activities were observed with Penicillium citrinum and Aspergillus fumigatus on PLA medium.

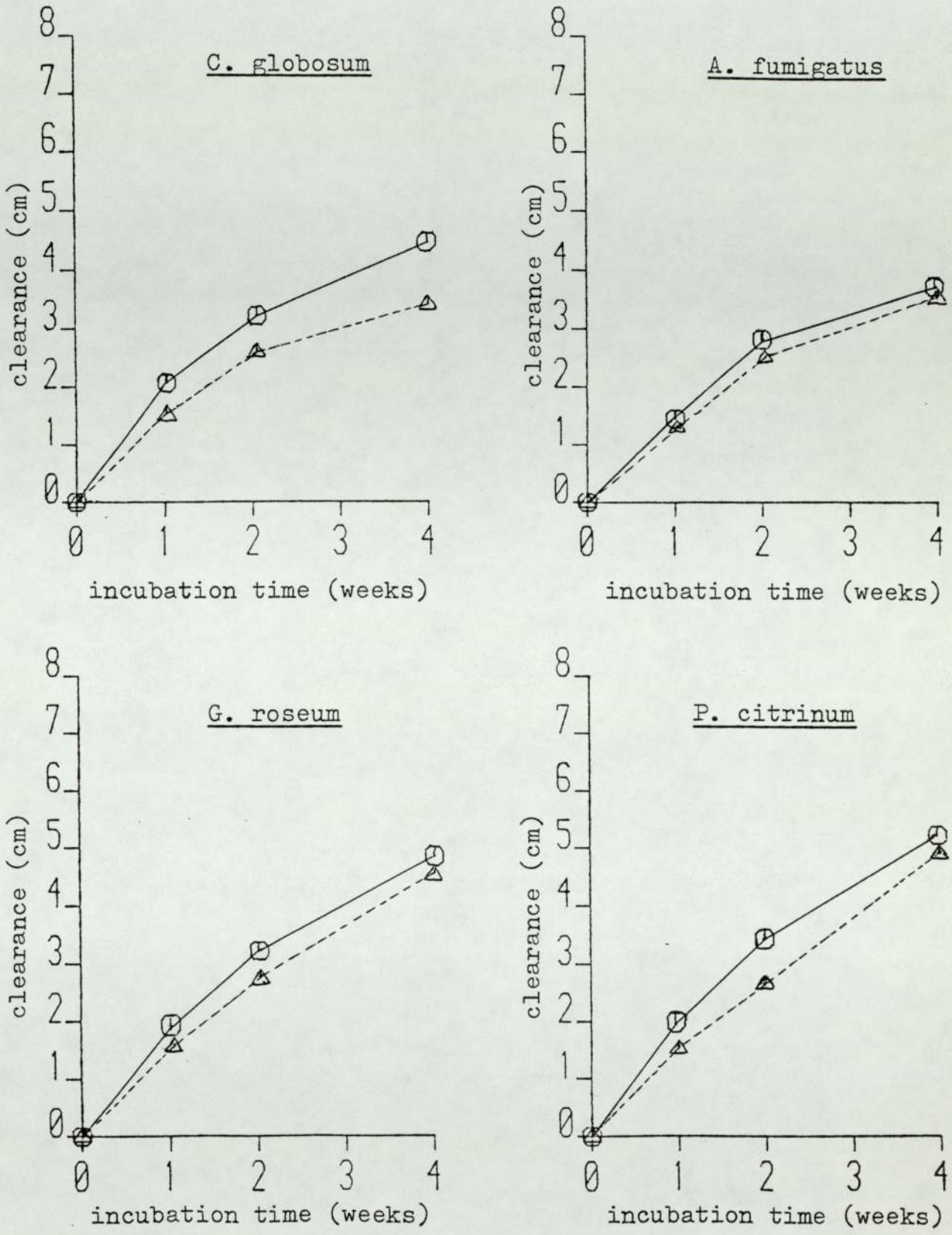


FIGURE 4.7

Amylase activities of test fungi on MEA (△-△) and PLA (○-○) media up to 4 weeks

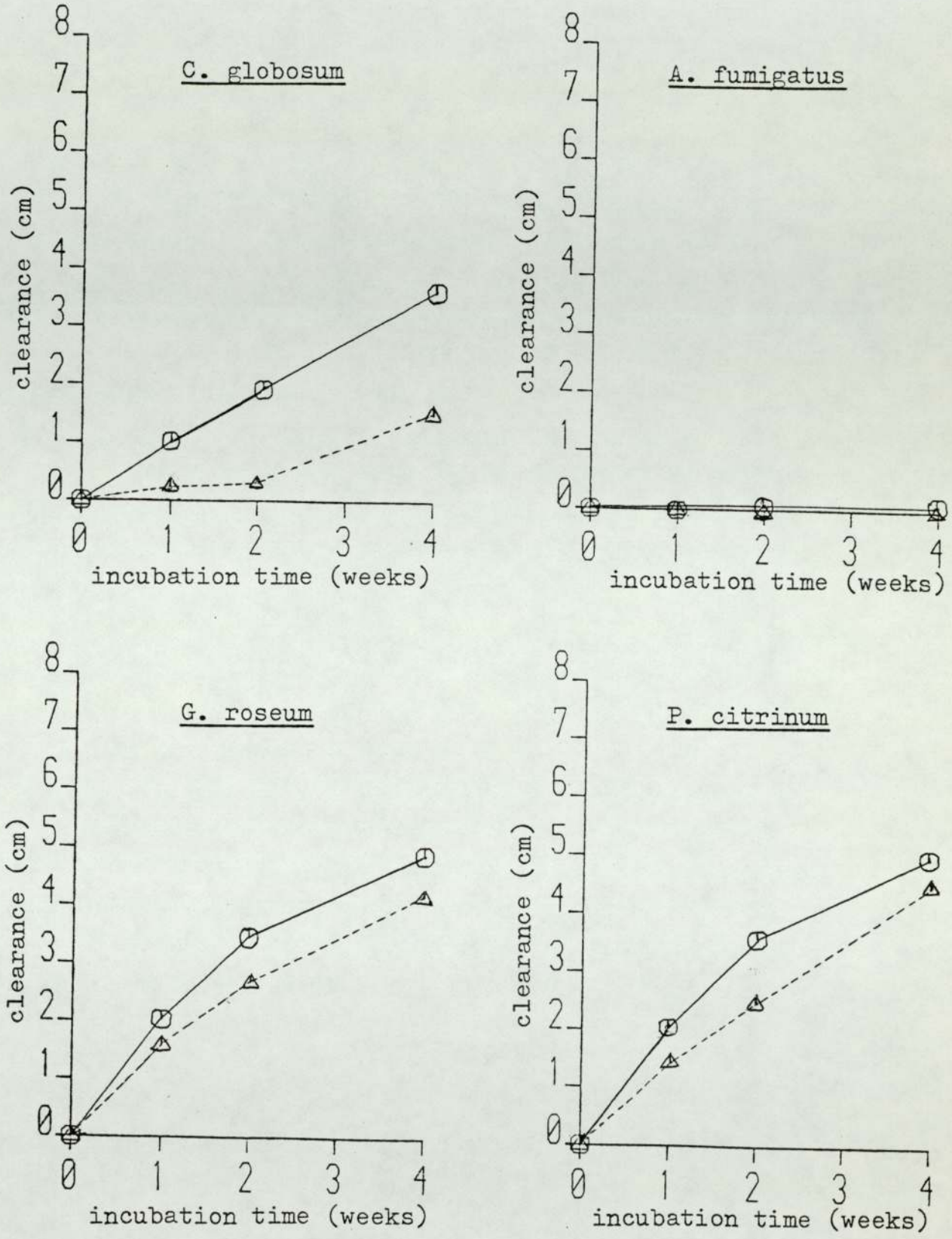


FIGURE 4.8

Protease activities of test fungi on MEA (Δ-Δ) and PLA (○-○) media up to 4 weeks

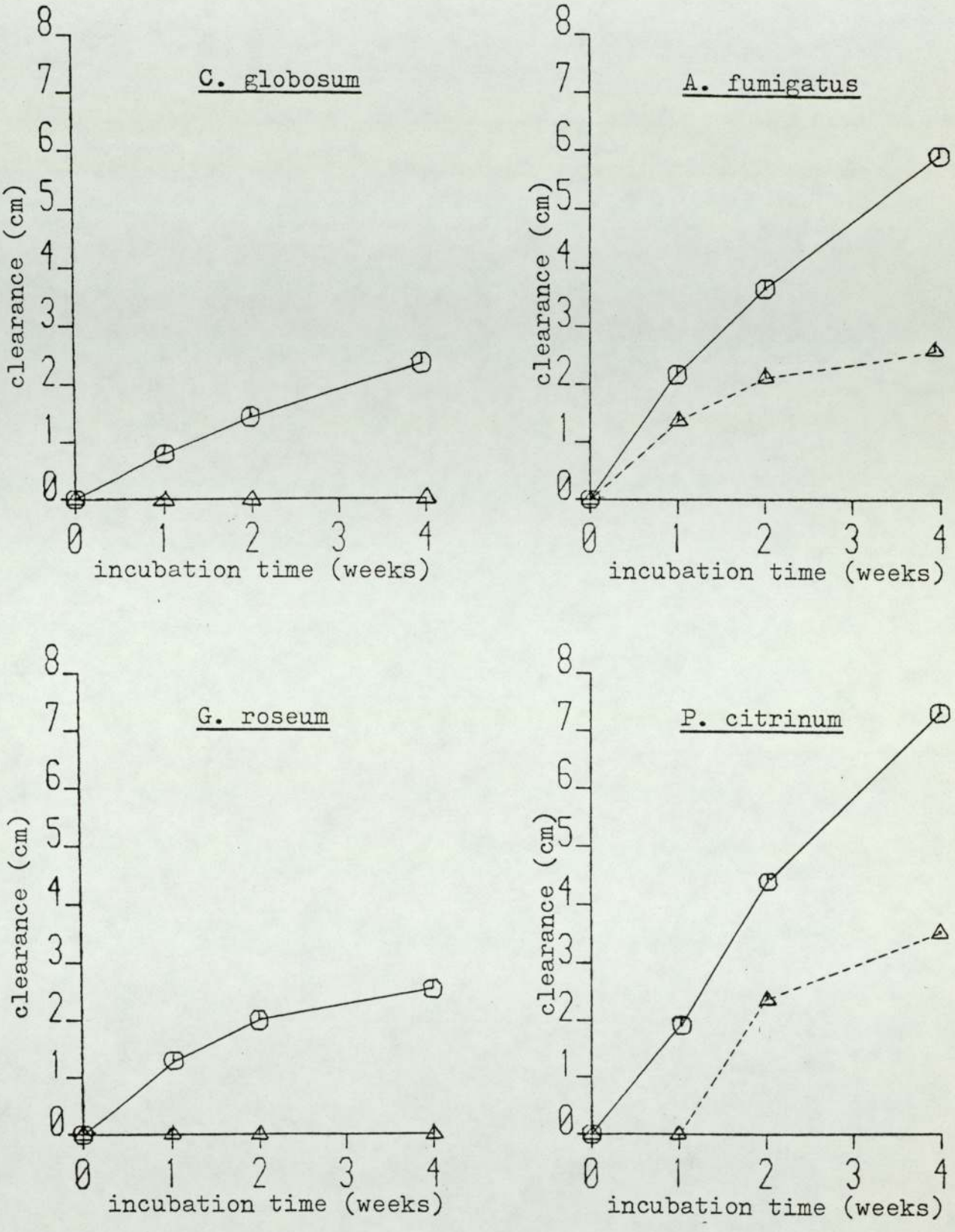


FIGURE 4.9

Esterase activities of test fungi on MEA (Δ-Δ) and PLA (○-○) media up to 4 weeks

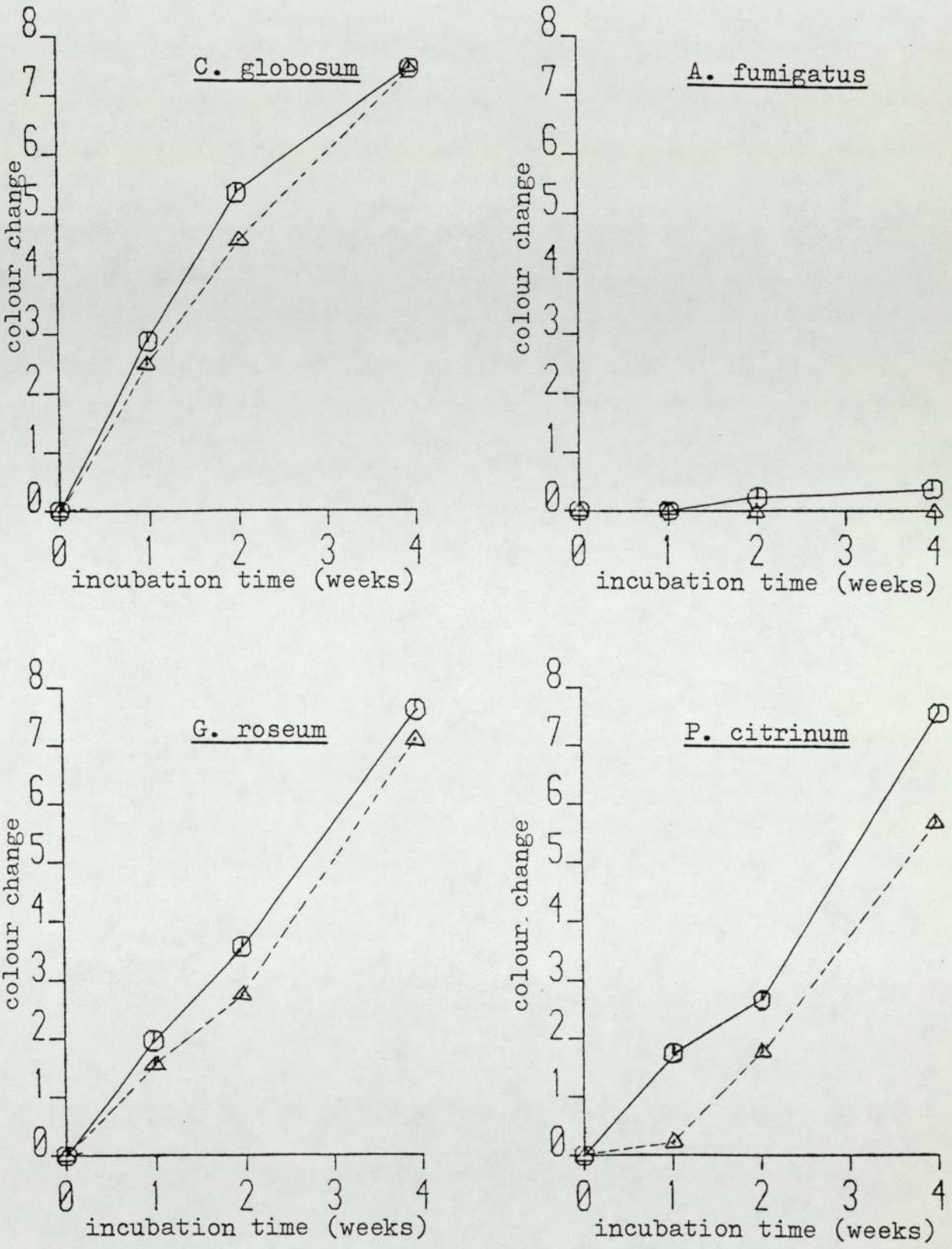


FIGURE 4.10

Urease activities of test fungi on MEA (Δ-Δ) and PLA (O-O) media up to 4 weeks

Another important observation made was that the growth and sporulation on MEA medium was extremely heavy when compared with the growth on PLA medium.

4.5 Discussion

Amylase, protease, esterase and urease activities were determined semiquantitatively by measuring the depth of clearance/colour change that developed beneath the growing cultures.

The clear zones in amylase activity determinations were caused by hydrolysis of starch by amylases into glucose. When dipped in iodine solution, starch molecules formed a blue complex whereas glucose showed no reaction leaving that zone clear.

In protease activity measurements, gelatin was hydrolysed by fungal proteases into smaller peptide and amino acid units. In saturated ammonium sulphate solution, unhydrolysed gelatin molecules precipitated making the medium opaque, but, smaller peptide and amino acid units remained unprecipitated, leaving that area clear.

Esterases catalysed hydrolysis of insoluble polyester molecules into soluble organic diacids and dialcohols. When this happened, the opacity caused by insoluble polyester molecules suspended in agar medium disappeared leaving a clear zone.

Ureases caused hydrolysis of urea molecules into ammonia and CO_2. The ammonia thus produced changed the pH of the medium, causing the colour change of bromothymol blue indicator from yellow to blue. The variations in intensity of blue colour were due to the amount of ammonia produced. When more ammonia was produced the intensity of blue colour increased. Diffusing ammonia reduced the distinction of margins.

In these methods, the size of test tubes and amounts of media can be varied according to the length of time the enzyme production has to be studied. The removal of agar columns in amylase and protease activity determinations can be avoided by using higher concentrations of starch and gelatin respectively to make media opaque, but, then the clearance will take a longer time.

In enzyme activity determinations of test fungi, amylase activity was studied as a reference. The results indicated that all test fungi were growing actively and producing enzymes freely and also justified selection of 0.5% mycological peptone + 0.3% lab-lemco powder medium as the basal medium.

Protease, esterase and urease activity results indicated that each test fungus was unique in its production of these enzymes and all of them varied considerably where enzyme activities were concerned.

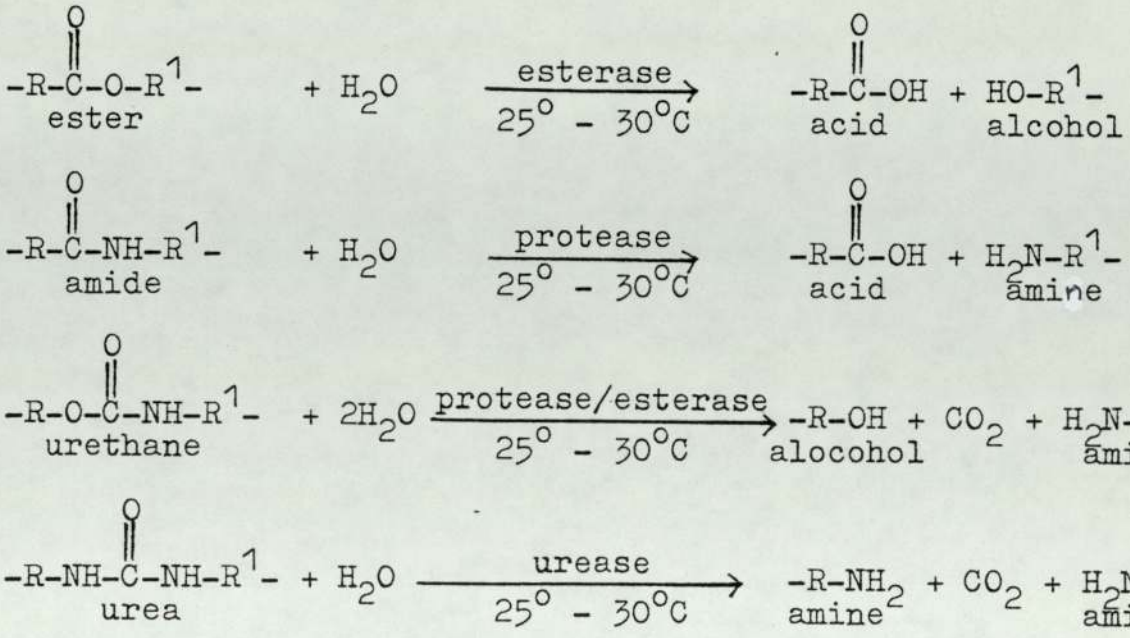
The variations in clearance of polyester medium by test fungi may be attributed to differences in the type of esterases produced by different fungi.

A probably explanation is that the esterases produced by Penicillium citrinum and Chaetomium globosum cause complete hydrolysis of polyester chains into diacids and dialcohols leaving the medium completely clear, whereas, esterases produced by Aspergillus fumigatus, Nigrospora spherica and Gliocladium roseum cause random hydolysis of polyester chains leaving small segments of partially-hydrolysed polyester units which are insoluble in water and thus cause incomplete clearance.

The very low or no protease and urease activities of Aspergillus fumigatus are very interesting where biodeterioration of polyurethanes is concerned. This indicates that biodeterioration of polyurethanes by Aspergillus fumigatus is not caused by proteases or ureases, but, only by esterases or a completely different enzyme system. Therefore, comparison of biodeterioration results of Aspergillus fumigatus in the following chapters with that of other test fungi which produce the whole range of enzymes tested may reveal interesting facts about the types of enzymes involved in biodeterioration of polyurethanes.

The importance of the enzymes tested in the process of biodeterioration of polyurethanes is that these enzymes have a tendency to catalyse hydrolysis of different types

of bonds at room temperature provided they have access to them. These reactions can be summarized as follows:-



In the study of effects of pH on enzyme production, all the fungi tested showed maximum amylase activity around neutral pH. The peak at pH 3 shown by Aspergillus fumigatus could be due to its maximum growth at that pH. This is further supported by the fact that both enzyme synthesis (by growing fungus) and enzyme activity are pH dependent.

The protease activity results show that proteases produced by all test fungi, except Aspergillus fumigatus which did not produce proteases, are active at acidic, neutral and basic pHs. It is a known fact that fungi can produce proteases active at acidic, neutral and basic pHs (Hagihara, 1960).

Esterase activities at different pHs varied considerably among test fungi. pH optima ranged from pH 5 to pH 7. This

indicated that there could be probably several different esterases produced by these fungi. The additional peak of Gliocladium roseum at pH 9 leads to the possibility of a single fungus producing different esterases. These are all tentative suggestions and more research with purified enzymes is needed before making any firm conclusions.

The urease activity maxima at low pH values (pH 4) shown by all test fungi can be explained as follows: Urease activity produces ammonia which increases the pH of the medium. Fungi can tolerate up to a maximum pH value. Therefore if fungi go on producing ureases irrespective of the pH increases of the surrounding medium, at one stage it will become self-destructive. Therefore, a control mechanism should exist within the fungus to avoid excess increase in pH due to urease activity. At acidic pHs (low pHs) fungi can afford to produce urease and allow the pHs to increase but at basic pHs they have to control urease activity for survival. So, when we test the product of urease activity, i.e. ammonia, to determine the real enzyme production a result like this is quite possible. Therefore to measure the actual production of urease, a method has to be devised to remove produced ammonia immediately.

A significant effect on enzyme activities of test fungi was observed with additional nutrients (malt extract). This effect is called "catabolite repression" and can be explained (Stadtman, 1970) as follows: Sometimes substrate-induced synthesis of catabolic enzymes is repressed when the energy

and carbon requirements of growth are amply supplied by a different catabolic process. For example when glucose is supplied in addition to a second substrate whose catabolism is under induced enzyme control, the glucose will be utilized preferentially, and as long as it is present, the enzymes required for catabolism of the second substrate will not be formed. This repressive effect need not result from glucose per se but may be attributed to one or more catabolites produced during the dissimilation of glucose. Moreover, the effect is not restricted to glucose; any one of several substrates that can support rapid growth may yield catabolites that repress the synthesis of inducible enzymes. From this explanation it shows that synthesis of proteases, esterases and ureases are induced by the substrate, at least partially.

The specificity of proteases, esterases and ureases are quite important in their capability of attacking polyurethanes. Esterases and proteases are mainly non-specific enzymes (Myers, 1969) and found to be quite similar in their action. For example certain proteolytic enzymes (trypsin and chymotrypsin types) have shown to hydrolyse appropriate carboxyl esters rapidly, by the same mechanism as an esterase. Some of the esterases, in turn have been shown to hydrolyse amide derivatives, acid anhydrides or even phosphate triesters.

This overlap in substrate specificity between the different classes of hydrolytic enzymes may be due, in some cases, to an indiscriminative bond-breaking mechanism which will act on any ester or amide that can approach the enzyme

activity centre closely.

Esterases have such a wide spectrum of attack that some have the characteristic feature of hydrolysing undissolved substrates (e.g. lipoprotein lipase).

Unlike proteases and esterases, ureases are said (Varner, 1969) to be so specific that even some substituted ureas were not hydrolysed by them.

Looking back at the wide variation in enzyme activities of test fungi it is quite interesting to see how they behave in deteriorating polyurethanes, in future chapters.

CHAPTER FIVE

PERCENTAGE WEIGHT LOSS AS A METHOD OF ASSESSING FUNGAL ACTIVITY ON POLYURETHANES

PERCENTAGE WEIGHT LOSS AS A METHOD OF ASSESSING FUNGAL ACTIVITY ON POLYURETHANES

The quantitative measurement of the adverse effects on properties of plastic films caused by the growth of micro-organisms may be assessed by a variety of methods and percentage weight loss is one of them.

Many workers have used this method to estimate microbial deterioration of plastic films such as polyvinyl chloride (Burgess and Darby, 1964; Hazeu, 1967; Hitz et al., 1967 and Wendt et al., 1970), ethyl vinyl acetate copolymers (Griffin and Mivetchi, 1976) and polyesters (Fields et al., 1974 and Diamond et al., 1975). In studies of biodeterioration of polyurethanes, weight loss determinations have only been used by Filip (1978) and Martens and Domsch (1981) to study the decomposition of polyurethanes in waste disposal systems.

In this study, the weight loss method was used as one of a series of experiments to estimate the effects of growth of test fungi on four polyester type and polyether type polyurethane elastomer films on a balanced nutrient agar medium (MEA) and on a basal medium (PLA) up to four weeks. Further to these the weight losses of one polyester type polyurethane on different media with gelatin, polyester and urea were compared with MEA and PLA media. The aim of this latter experiment was to observe whether there was any significant induction or inhibition of the test fungi to produce a particular enzyme/s by the above substances to

affect the rates of deterioration of test polyurethane.

5.1 Materials and methods

The following polyurethanes were used (appendix 2):-

1. Yellow soft polyester type polyurethane elastomer.

2. Red medium polyester type polyurethane elastomer.

3. White hard polyester type polyurethane elastomer.

4. Red hard polyester type polyurethane elastomer.

5. Yellow hard polyether type polyurethane elastomer.

Thin sheets (30cm x 30cm x 0.05cm) of each elastomer were prepared by a polyurethane manufacturing company. No chemical structures were disclosed because all were commercial products.

5.1.1 Procedure for assessment of fungal attack on polyurethanes based on percent weight loss

The test pieces were cut with a dumbell-shaped die (length = 5cm; breadth = 0.35 cm; gauge length = 3.0cm) and were suitably marked with permanent ink. These were dried over silica gel in a vacuum desiccator (pressure = 35cm Hg) for 24 hours to a constant weight and were weighed

accurately up to 0.1mg. The samples were then sterilized as follows: The pieces were washed thoroughly in a dilute washing up solution (1ml washing up liquid in 1 litre solution), rinsed in several changes of water and then dried between two sheets of clean blotting paper. The dried pieces were placed in a formalin vapour tank (figure 5.1) for 3 hours, removed aseptically into bottles containing sterile distilled water and then soaked overnight to remove any diffused formalin. Finally the test pieces were washed in six changes of sterile distilled water.

The samples were drained and placed in sets of two on the cooled surfaces of MEA and PLA plates (25ml medium per plate). The plates were inoculated with 0.5ml spore suspensions of Gliocladium roseum, Chaetomium globosum, Penicillium citrinum and Aspergillus fumigatus and were incubated at 30 ± 1°C after sealing them in polythene bags to avoid desiccation. After incubation for 1, 2 or 4 weeks, the test pieces were very carefully removed, washed gently in water and soaked overnight to remove any soluble substances, and finally air-dried for two days. Before taking the final weights samples were dried over silica gel in a vacuum desiccator to a constant weight. The percentage weight losses were calculated as:

$$\% \text{ weight loss} = \frac{\left(\text{initial weight of the sample (g)} - \text{final weight (g)}\right)}{\text{initial weight (g)}} \times 100\%$$

Each experiment was done with six replicates. Controls were

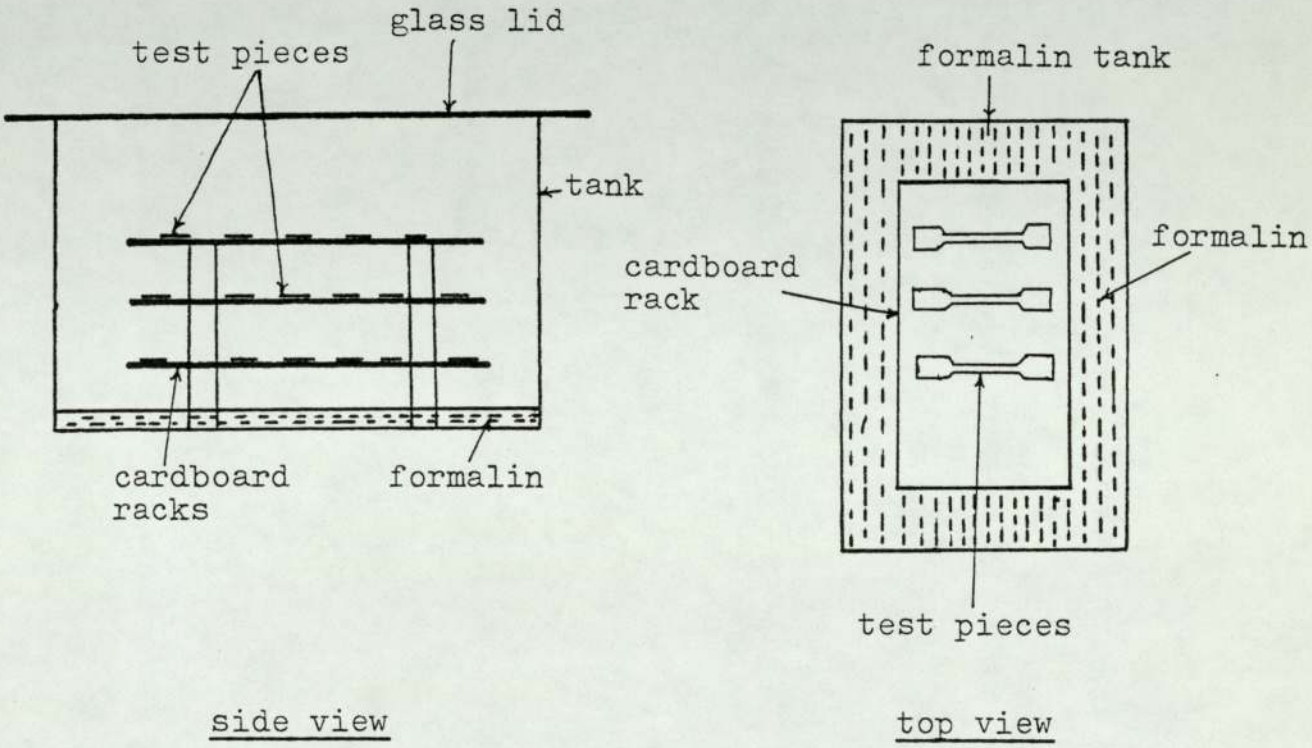


FIGURE 5.1

Formalin vapour tank used to sterilize the test polyurethane pieces

(N.B. cardboard shelves were previously saturated with formalin vapour to effect a complete sterilization of the material on both sides at the same time.)

set up without inoculation.

To determine the effects of gelatin, urea and polyester supplements in the medium, dumbell-shaped test pieces from the white hard polyester type polyurethane were cut, labelled, weighed and sterilized as before. The samples were plated in sets of two onto cooled surfaces of gelatin agar, urea agar, polyester agar, MEA and PLA plates (appendices 1h, 1j, 1i, 1a and 1l respectively) and the treatment procedure carried out as above with the exception that the incubation period was ten days.

5.2 Results

Figures 5.2 to 5.6 contain data obtained on percent weight losses of yellow soft, red medium, white hard and red hard polyester type polyurethane elastomers and yellow hard polyether type polyurethane elastomer respectively with four fungi plotted versus the time of incubation. Each point represents the mean of six replicates.

All polyester type polyurethanes showed (figures 5.2 to 5.5) varying degrees of weight losses with different fungi whereas the polyether type polyurethane indicated (figure 5.6) relatively insignificant weight losses with different fungi, except with Chaetomium globosum, where a weight increase was observed on MEA.

The weight losses of all four polyester type

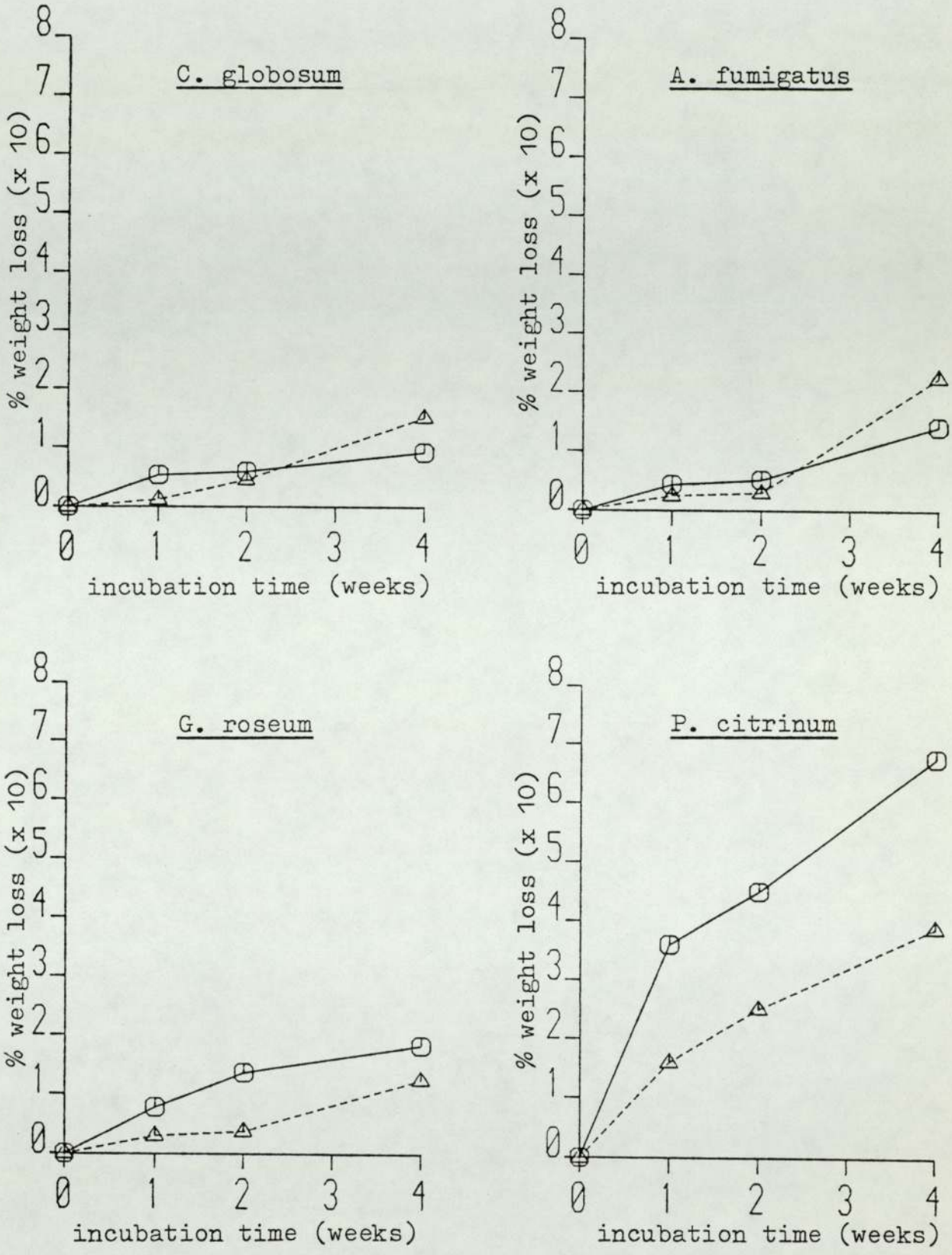


FIGURE 5.2

Percent weight loss of yellow soft polyester polyurethane when incubated with test fungi up to 4 weeks on MEA (△-△) and PLA (○-○) media

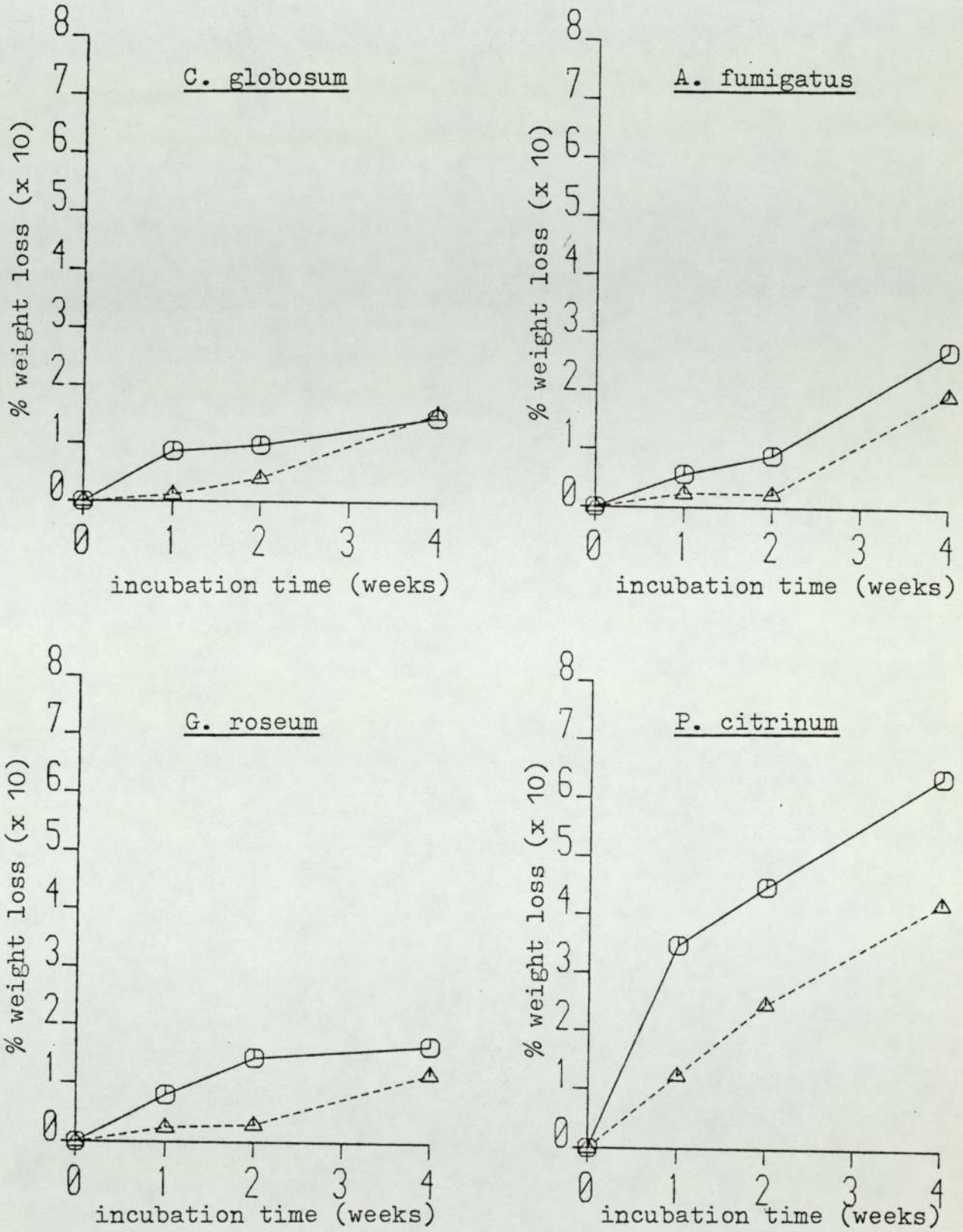


FIGURE 5.3

Percent weight loss of red medium polyester polyurethane when incubated with test fungi up to 4 weeks on MEA (△-△) and PLA (○-○) media

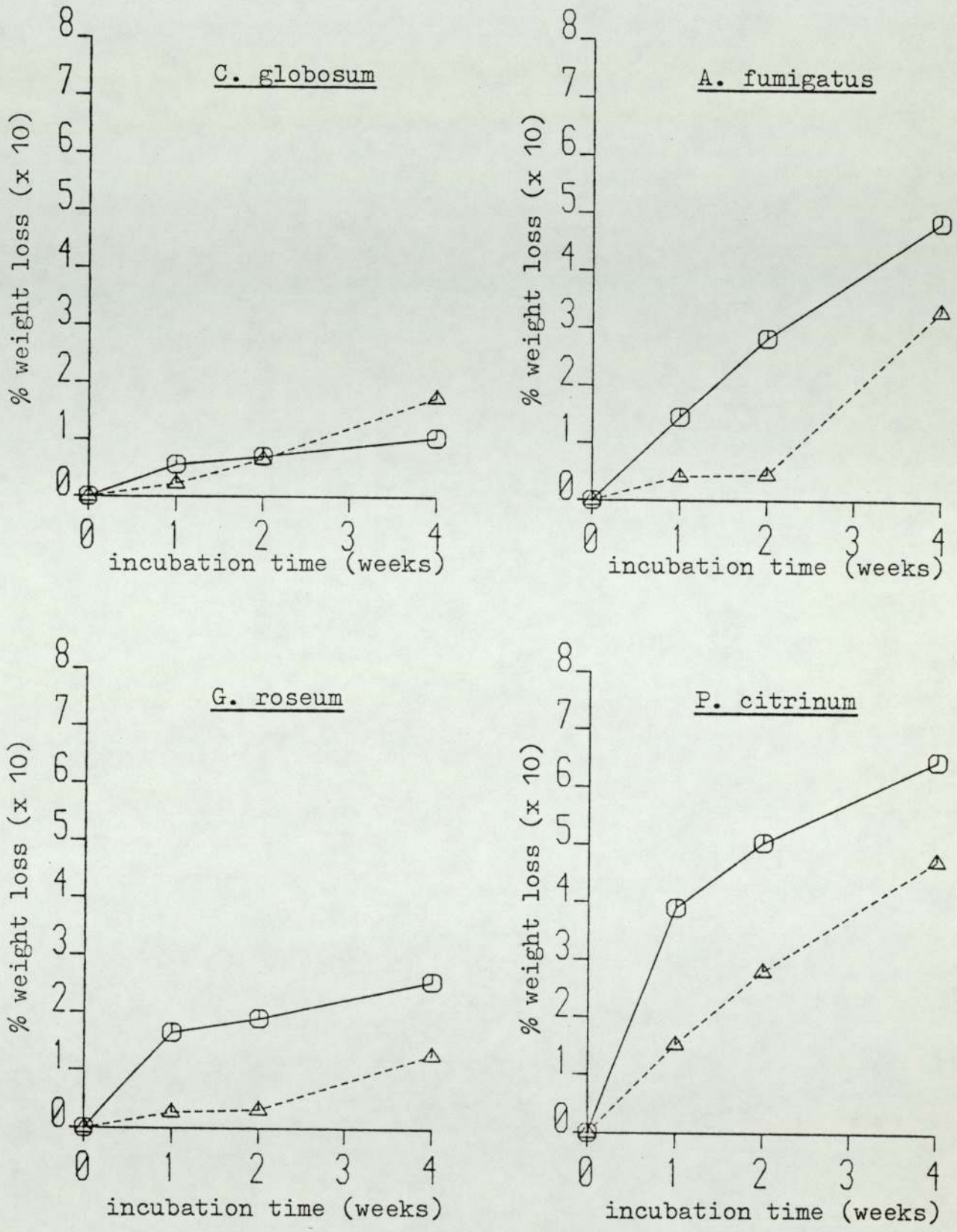


FIGURE 5.4

Percent weight loss of white hard polyester polyurethane when incubated with test fungi up to 4 weeks on MEA (△-△) and PLA (○-○) media

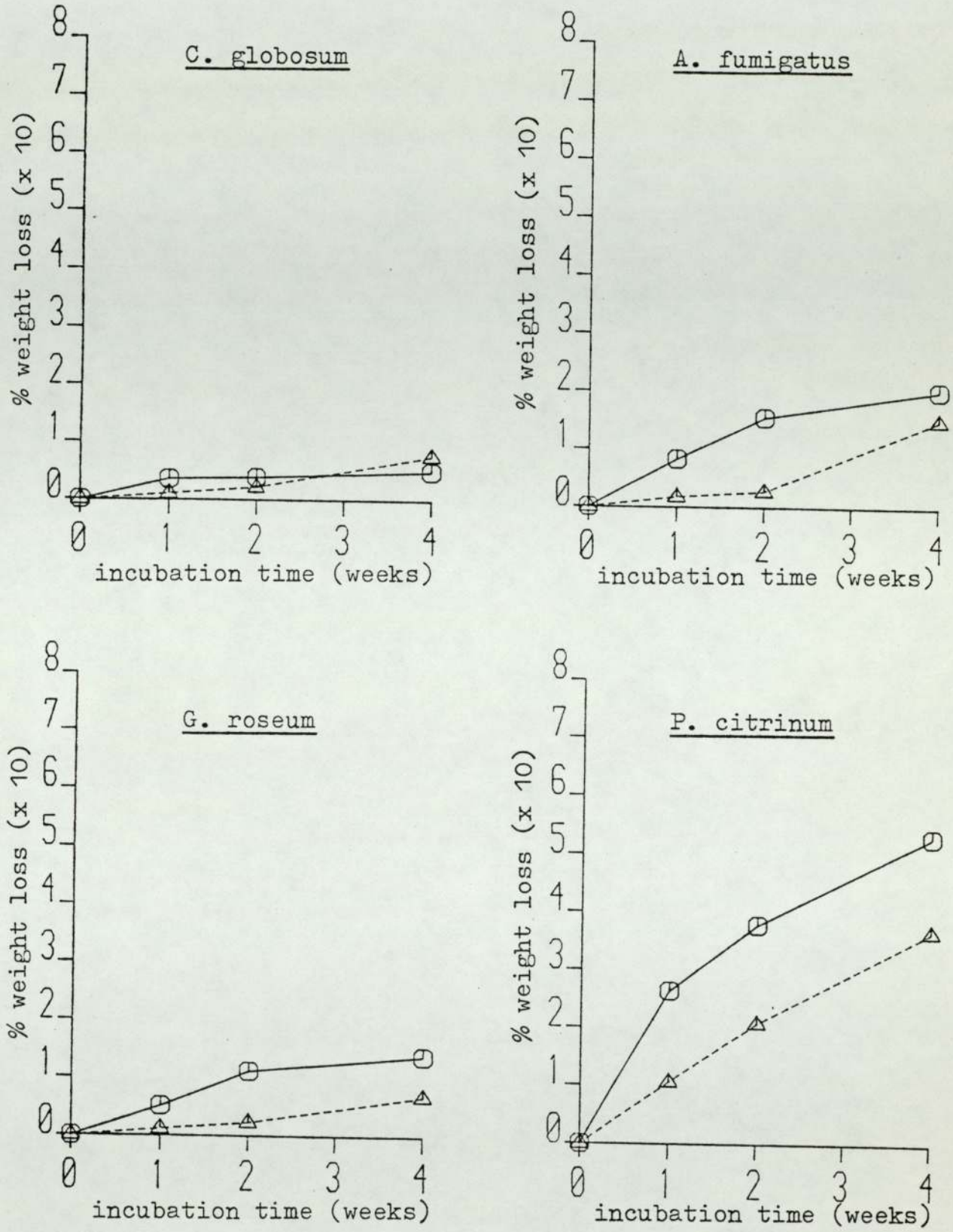


FIGURE 5.5

Percent weight loss of red hard polyester polyurethane when incubated with test fungi up to 4 weeks on MEA (Δ-Δ) and PLA (O-O) media

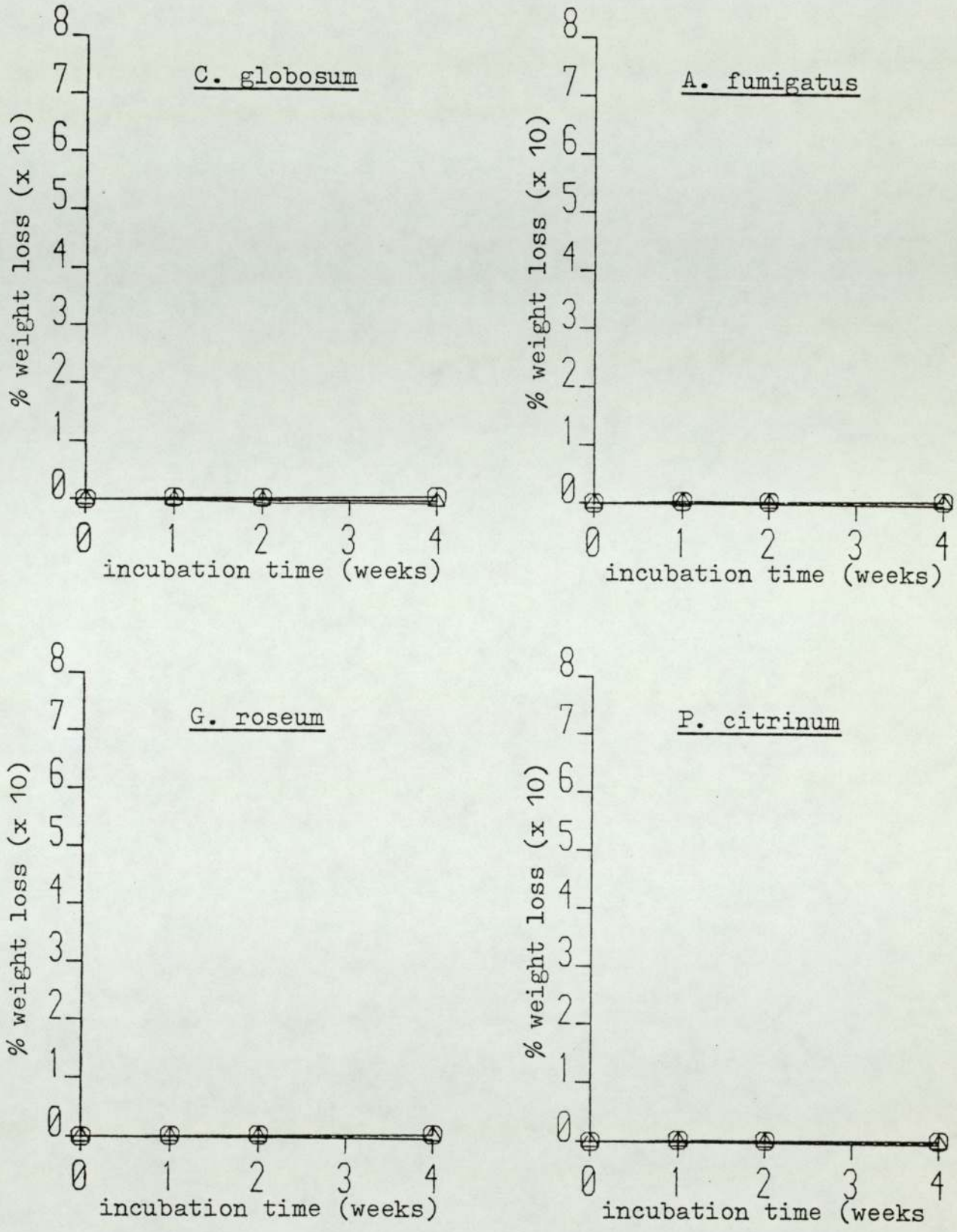


FIGURE 5.6

Percent weight loss of yellow hard polyether polyurethane when incubated with test fungi up to 4 weeks on MEA (△-△) and PLA (○-○) media

polyurethanes gradually increased with time of incubation. The effects of PLA and MEA media on weight losses showed interesting results. The initial weight losses (up to 1 - 2 weeks incubation) on PLA were significantly higher than on MEA with all test fungi, but, the final weight losses (after 4 weeks incubation) indicated that the gap between weight loss values on PLA and MEA had considerably reduced with Gliocladium roseum and Aspergillus fumigatus, and with Chaetomium globosum the weight losses on MEA were greater than on PLA. Only with Penicillium citrinum the gap between PLA and MEA remained almost unaltered.

The maximum weight losses caused by different fungi on the four test polyester type polyurethanes are summarized in table 5.1.

In general, the highest and lowest weight losses were observed with white hard and red hard polyester type polyurethanes respectively. Penicillium citrinum showed extremely high weight losses in the region of 50% - 70% with all polyester type polyurethanes. The other fungi showed maximum weight losses with all polyester polyurethanes in decreasing order (Aspergillus fumigatus > Gliocladium roseum > Chaetomium globosum), but, all these could be broadly catagorized in one group where weight losses were concerned. In all cases, the higher weight losses were indicated by shrinking and even complete embrittlement. To exhibit these effects a single example of deterioration of yellow soft polyester polyurethane by Penicillium citrinum is shown in figure 5.7.

Test Polyester type Polyurethane	G. roseum		C. globosum		P. citrinum		A. fumigatus	
	Medium	% weight loss	Medium	% weight loss	Medium	% weight loss	Medium	% weight loss
Yellow soft	PLA	18.23	MEA	15.19	PLA	67.62	MEA	22.61
Red medium	PLA	16.40	MEA	15.25	PLA	63.76	PLA	27.09
White hard	PLA	25.49	MEA	17.29	PLA	64.52	PLA	48.45
Red hard	PLA	13.77	MEA	7.68	PLA	52.97	PLA	20.18

TABLE 5.1

MAXIMUM % WEIGHT LOSSES CAUSED BY TEST FUNGI ON POLYESTER TYPE POLYURETHANES

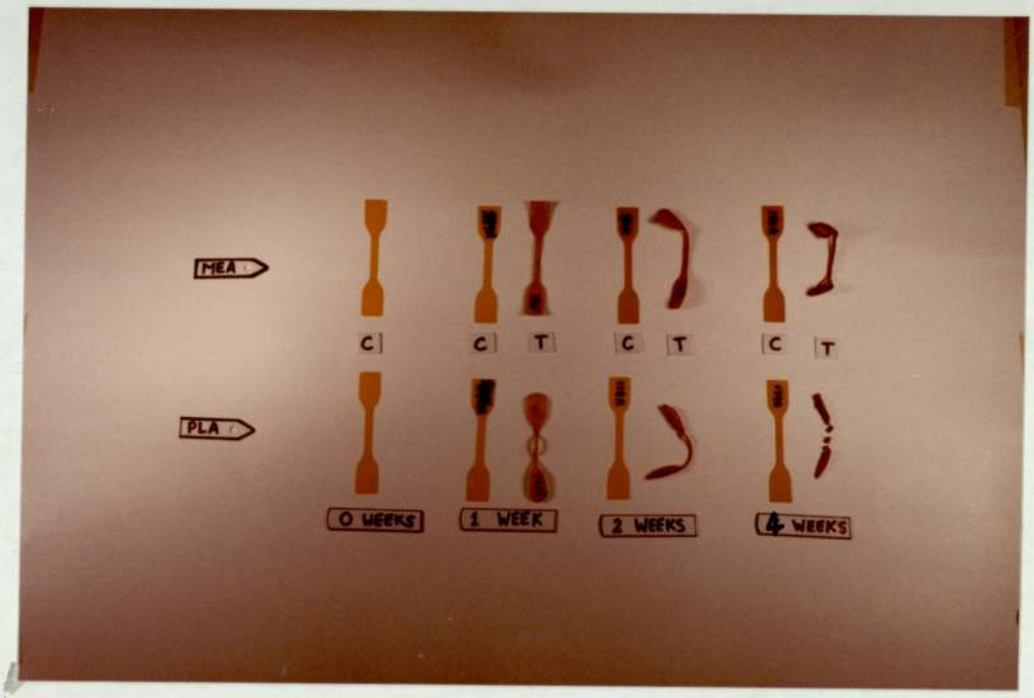


FIGURE 5.7

Biodeterioration of yellow soft polyester polyurethane when incubated with P. citrinum on MEA and PLA media up to 4 weeks

C = control

T = test piece

Another interesting observation was the degree of growth of fungi on different media. On MEA plates a dense growth was observed on the medium as well as on the material, but on PLA plates there was a sparse growth on the medium and a relatively dense growth on the material. This is clearly shown in figure 5.8 where growth of test fungi on red medium polyester type polyurethane after 7 days incubation was taken as a reference.

The means and standard deviations of weight loss results were calculated and are presented in tables 5.4 to 5.8 (appendix 3).

Table 5.2 contains the data obtained from weight losses from the white hard polyester polyurethane with four test fungi on different nutrient agar media after 10 days incubation.

As gelatin, polyester and urea supplements were added to the basal medium (PLA), the weight losses on these media were compared with the results on PLA. To make this comparison simple, changes of weight losses relative to weight losses observed on PLA are summarized in table 5.3.

All fungi showed reductions in relative weight losses on polyester medium. On urea medium Gliocladium roseum showed an increase and the remainder showed decrease in relative weight losses. A significant decrease in relative weight losses on urea was observed with Penicillium citrinum.

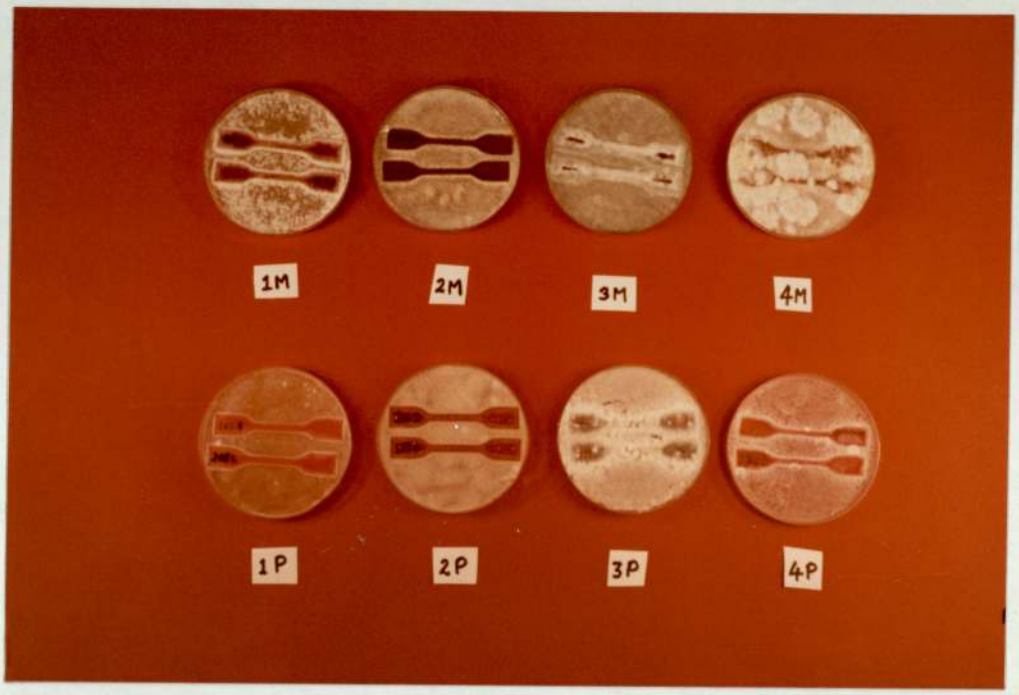


FIGURE 5.8

Amounts of biomass produced by test fungi when plated on MEA (M) and PLA (P) media up to 7 days with red medium polyester polyurethane

1 = G. roseum

2 = C. globosum

3 = P. citrinum

4 = A. fumigatus

TABLE 5.2

PERCENT WEIGHT LOSSES OF WHITE HARD POLYESTER POLYURETHANE WHEN INCUBATED WITH TEST FUNGI ON DIFFERENT NUTRIENT MEDIA

medium/pH	% WEIGHT LOSS			
	G. roseum	C. globosum	P. citrinum	A. fumigatus
PLA (5.7)	22.61 ± 1.65	7.09 ± 0.26	46.46 ± 6.17	25.56 ± 2.20
PEA (5.6)	11.07 ± 1.30	4.60 ± 1.42	19.38 ± 1.72	17.50 ± 1.89
UA (5.7)	25.74 ± 2.96	5.10 ± 0.34	1.85 ± 0.24	13.11 ± 2.08
GA (5.4)	23.99 ± 2.05	6.98 ± 0.59	60.27 ± 0.91	33.83 ± 2.07
MEA (5.4)	4.46 ± 0.30	6.28 ± 0.28	23.94 ± 1.34	13.50 ± 2.28

N.B. PEA = polyester agar
UA = urea agar
GA = gelatin agar
(appendices 1i, 1j, 1h)

TABLE 5.3

RELATIVE CHANGES IN % WEIGHT LOSSES ON DIFFERENT MEDIA WITH RESPECT TO % WEIGHT LOSSES ON PLA MEDIUM

medium	RELATIVE CHANGES IN % WEIGHT LOSSES			
	G. roseum	C. globosum	P. citrinum	A. fumigatus
PLA	0.00	0.00	0.00	0.00
PEA	-11.54	-2.49	-27.08	- 8.06
UA	+ 3.13	-1.99	-44.61	-12.45
GA	+ 1.38	-0.11	+13.81	+ 8.27
MEA	-18.15	-0.81	-22.52	-12.06

With the addition of gelatin to the medium all fungi except Chaetomium globosum showed increases in relative weight losses. On MEA all fungi showed decrease in relative weight losses.

5.3 Discussion

The percentage weight loss results clearly indicate that the polyester type polyurethanes tested are susceptible to fungal attack whereas the polyether type is shown as completely resistant.

The slight increase in weight observed on polyether type polyurethane in the presence of Chaetomium globosum on MEA cannot be due to migration of some components from the medium into the polymer because it was not observed with control pieces without inoculum on the same medium. Therefore the other possibility is migration of some exudate produced by Chaetomium globosum into the polymer. However, a fuller explanation remains to be sought.

An explanation for the variations in weight losses of polyester polyurethanes on MEA and PLA with incubation time is as follows: MEA provides a readily available source of nutrients for the growth of fungi. This probably causes a catabolite repression (Chapter 4) which in turn inhibits the production of inducible polyurethane degrading enzymes until the readily available nutrient source is depleted. But, due to readily available nutrients fungi grow rapidly in a luxuriant manner although they are unable to degrade the polymer in the early stages of incubation. On the other

hand, in PLA medium the nutrients are limiting and therefore there is no catabolite repression. To survive these fungi initially resort to utilization of the polyurethane pieces resulting in initial high weight losses. This probably explains the relatively low weight losses observed on MEA in the early stages of incubation, although there is good, heavy growth.

When the readily available nutrients on MEA are depleted, the fungi resort to the only other source of nutrient, i.e. the polyester polyurethane pieces. By the time the fungi are well established, a heavy demand exists for nutrients, and the polymer is utilized quite rapidly. In contrast to this, on PLA medium, the growth of fungi is restricted from the beginning and even at later stages the demand for nutrients is quite static. For these reasons, at later stages in this experiment the weight losses on MEA increase rapidly and narrow the gap with the weight losses on PLA.

Weight loss of a water insoluble polymer in a microbial environment is caused by splitting of the polymer chains by the enzymes produced by the microorganism into small, water-soluble units which can be utilized by the microorganism or which will be leached off to the surrounding environment. If this splitting of polymer chains is regular, then all the breakdown products will be small enough to be soluble in water, but, if the splitting is at random, then only a part of the breakdown products will be small enough to be soluble in water. Therefore, according to this hypothesis, an enzyme

system with regular attack will contribute more towards weight losses of a polymer than a similar enzyme system with random attack.

Using this hypothesis, the weight losses caused by different test fungi can be explained. Penicillium citrinum showed extremely high weight losses with all four types of polyester polyurethanes. Therefore, it should possess an extremely effective enzyme system of breaking the polyester polyurethane chains into small water-soluble units, similar to the enzyme system with regular splitting described in the hypothesis. The remainder which show comparatively low weight losses may possess similar enzymes but at low concentrations or may have an entirely different system of random splitting which do not contribute to weight losses as much as the above mentioned system as explained in the hypothesis.

The effects of different substances added to the basal medium showed quite interesting results. The decrease in relative weight losses on MEA can be explained as catabolite repression of polyurethane degrading enzyme system by the abundant sugars present in the medium. The relative decrease in weight losses on polyester agar can be due to competitive inhibition by the polyester units present in the medium. The increase in relative weight losses on gelatin agar may be attributed to the induction of a 'polyester polyurethane degrading enzyme system' because otherwise it should act as another available source of nutrients to fungi, thus decreasing the relative weight losses. With urea in the

medium only Gliocladium roseum showed a slight increase in weight losses. The significant decrease in relative weight losses with Penicillium citrinum and Aspergillus fumigatus can only be explained as inhibition of the 'polyester polyurethane degrading enzyme system' because urea cannot provide a nutritive carbon source for the growth of fungi. The changes observed with Chaetomium globosum are relatively insignificant because it takes more than ten days for this fungus to show its effects.

Sterilization of the test pieces is very important for obvious reasons. Contamination of test plates and even controls after certain sterilization procedures were reported in literature in several instances. In the first inter-laboratory experiment on biodeterioration of plastics (Hazeu, 1967) it was reported that control samples sterilized either by 95% ethanol for 5 seconds or ethylene dioxide were contaminated by fungi and other microorganisms. Pankhurst et al. (1971), in their studies on biodeterioration of polymeric materials associated with protective systems for buried pipe lines, recorded that some test plates and even some control plates sterilized with 1% $HgCl_2$ were contaminated with fungi.

In the present study, an attempt was made to find a suitable sterilizing method because the polyurethanes tested were unable to be sterilized by autoclaving due to possible hydrolysis. Initial experiments using standard sterilization methods (95% ethanol, 70% ethanol, 1% $HgCl_2$ and 2% H_2O_2) did not provide sufficient degrees of sterilization. After many trial experiments, it was found that the sterilization

procedure described in materials and methods was quite efficient and gave almost 100% results.

As the same samples used for weight loss determinations will be used for physico-mechanical testing in the next chapter, it will be very interesting to see how the enzymes produced by these fungi affected the physico-mechanical properties of the test polyurethanes.

CHAPTER SIX

PHYSICO-MECHANICAL STUDIES ON BIODETERIORATION OF POLYURETHANES BY TEST FUNGI

PHYSICO-MECHANICAL STUDIES ON BIODETERIORATION OF POLYURETHANES BY TEST FUNGI

The effects of fungi on polyurethanes can be measured in terms of a number of different properties. The importance of any one of these properties depends upon the application being considered for the product. As the polyurethanes tested in this study are commercial elastomers, changes in tensile strength and percent ultimate elongation are general measures of the extent of degradation.

Kaplan et al. (1968) studied the changes in tensile strength, percent ultimate elongation and modulus at hundred and three hundred percent elongation of polyester type and polyether type polyurethanes buried in soil for up to three months. Use of an ultraviolet inhibitor and a hydrolysis inhibitor in the polyester type polyurethane did not prevent the changes in physical properties in the elastomer during soil burial. Therefore, by process of elimination they showed that the prime factor in causing the physical changes in the elastomers during soil burial was microbial activity.

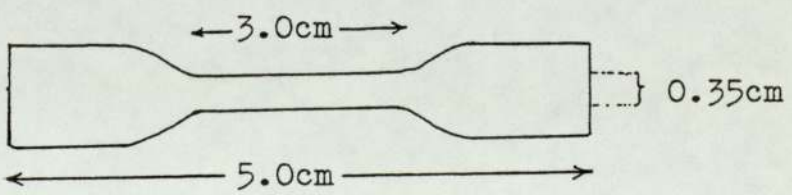
Changes in tensile strength was used as a technique by Hedrick and Crum (1968) to study the effects of jet fuel microbial isolates on a polyurethane foam used as a baffling material in aircraft fuel tanks. They observed that Cladosporium resinae caused maximum deterioration in fuel + salts medium where tensile strength decreased from 25 psi to 6 psi after 60 days at 30°C.

In the study described below, tensile strength and percent ultimate elongation /which is the percent ratio between maximum elongation of the test sample before breaking and the original length of the sample between jaws of the tensiometer (gauge length)/ have been studied to understand the contribution of test fungi towards the biodeterioration of polyurethane elastomers.

6.1 Materials and methods

The dumbell pieces used for weight loss measurements (Chapter 5) to estimate the effects of growth of test fungi on four polyester type and one polyether type polyurethanes on MEA and PLA, were used for tensile strength and percent ultimate elongation measurements. Further to these, the dumbell pieces used to study the weight losses of white hard polyester polyurethane ondifferent media with gelatin, polyester and urea as well as MEA and PLA, were tested for changes in tensile strength and percent ultimate elongation.

All pieces were conditioned at 60 ± 3% relative humidity at 20° ± 1°C in a humidity and temperature controlled room for two weeks. These test pieces were cut using an E type dumbell cutter to the following dimensions:-



length = 5.0cm; breadth = 0.35cm; gauge length = 3.0cm

Tensile tests were carried on an Instron Tensile Tester (model TMSM) (figure 6.1) using a crosshead speed of 10cm per minute. The relevant data was displayed on a chart recorder. The chart speed was 10cm per minute.

Tensile strength and percent ultimate elongation were calculated from the following equations, using the data obtained from the stress-strain curves (Setoudeh, 1981).

$$\text{tensile strength} = \frac{\text{force at break}}{\text{thickness x width}}$$

$$\text{percent ultimate elongation} = \frac{\text{chart length x cross-head speed}}{\text{chart speed x gauge length}} \times 100\%$$

Each experiment was done with six replicates

6.2 Results

Figures 6.2 - 6.6 contain data obtained from tensile strength measurements of yellow soft, red medium, white hard and red hard polyester type polyurethane elastomers and yellow hard polyether type polyurethane elastomer respectively, with four test fungi plotted versus the time of incubation. Each point represents the mean of six replicates.

All polyester type polyurethanes showed (figures 6.2 - 6.5) varying degrees of tensile strength losses with different fungi, whereas, with polyether type polyurethane, the variations with different fungi were insignificant (figure 6.6).

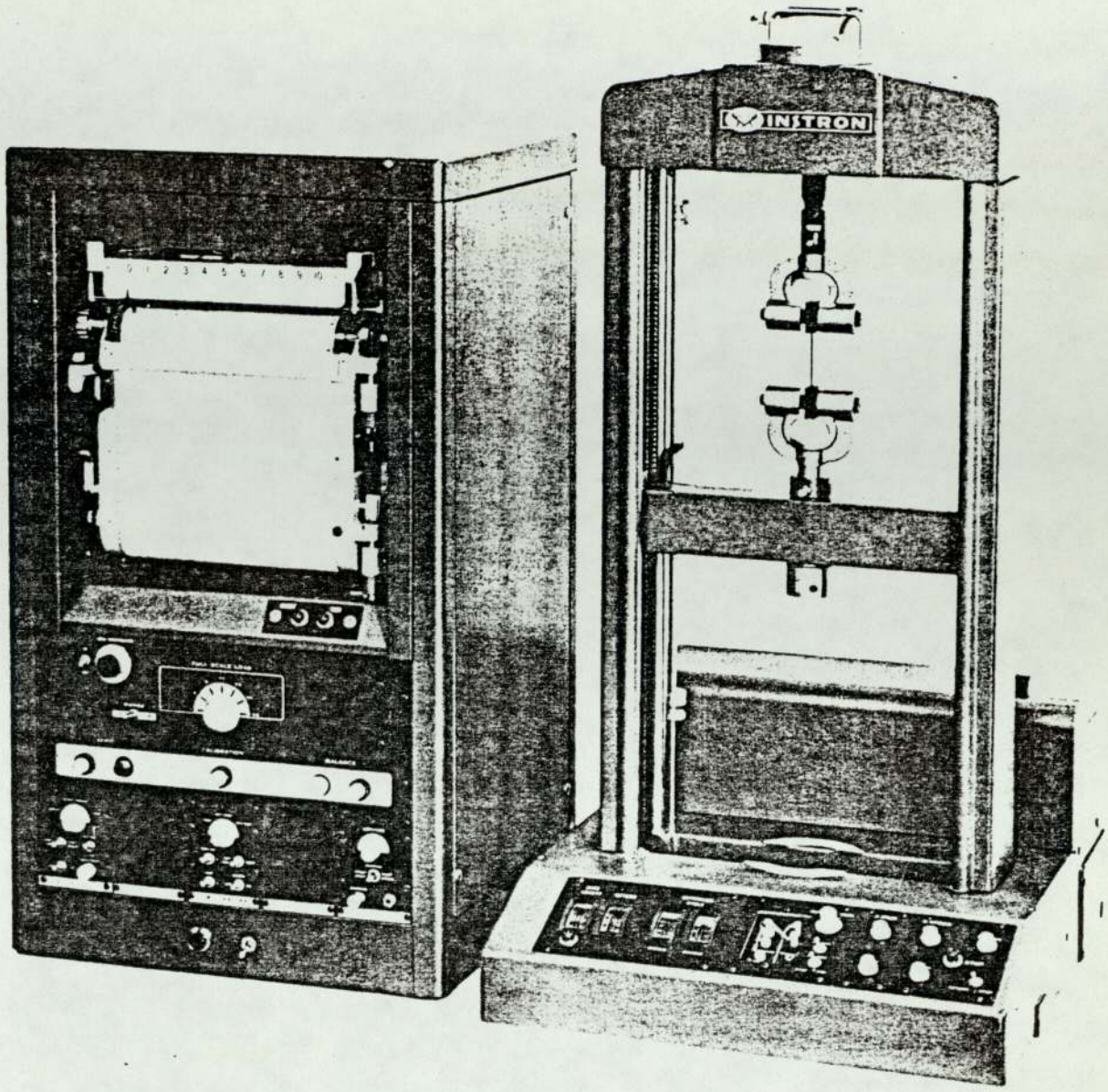


FIGURE 6.1

Instron Tensile Tester (model TMSM)

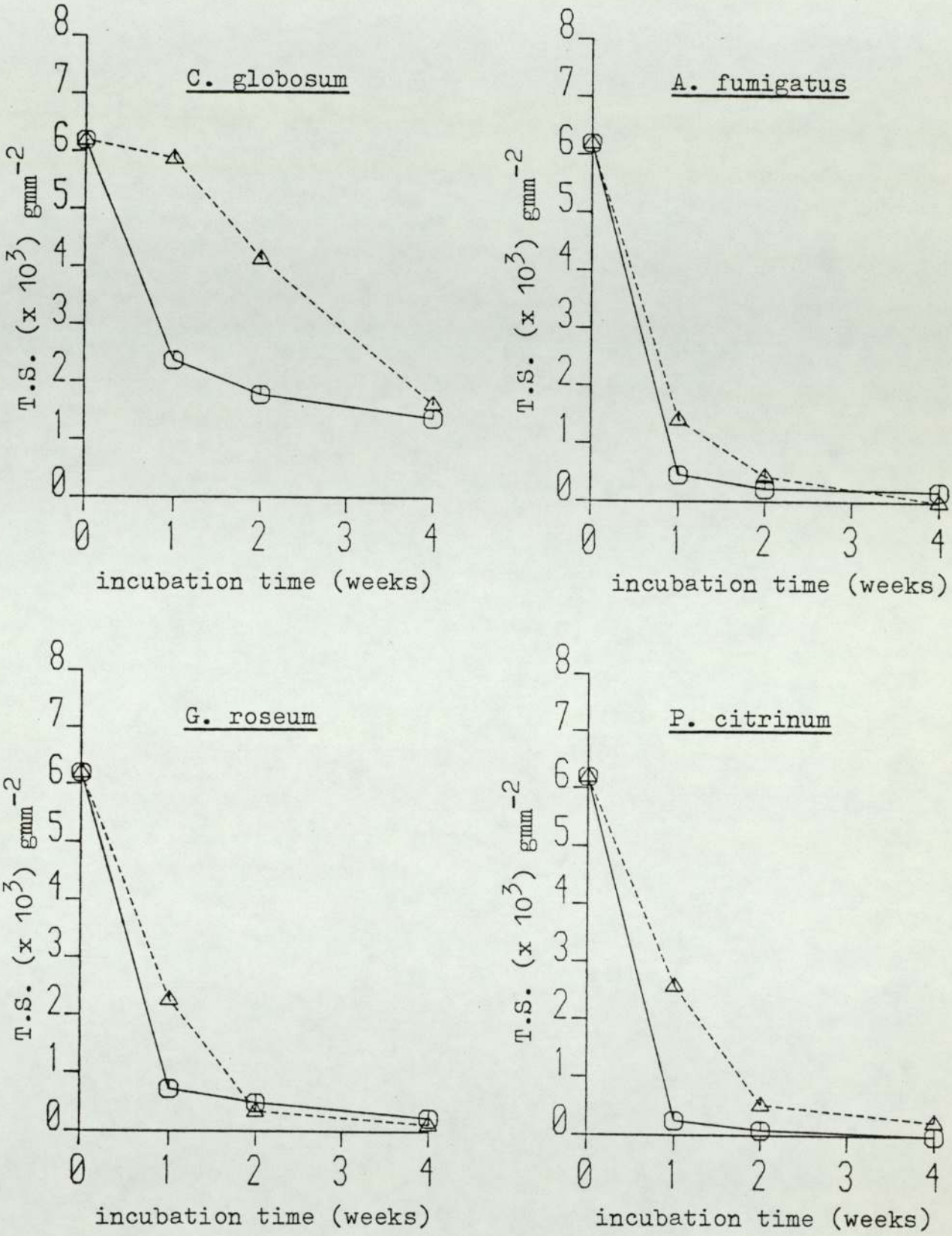


FIGURE 6.2

Tensile strengths (T.S.) of yellow soft polyester polyurethane when incubated with test fungi on MEA (Δ-Δ) and PLA (○-○) media

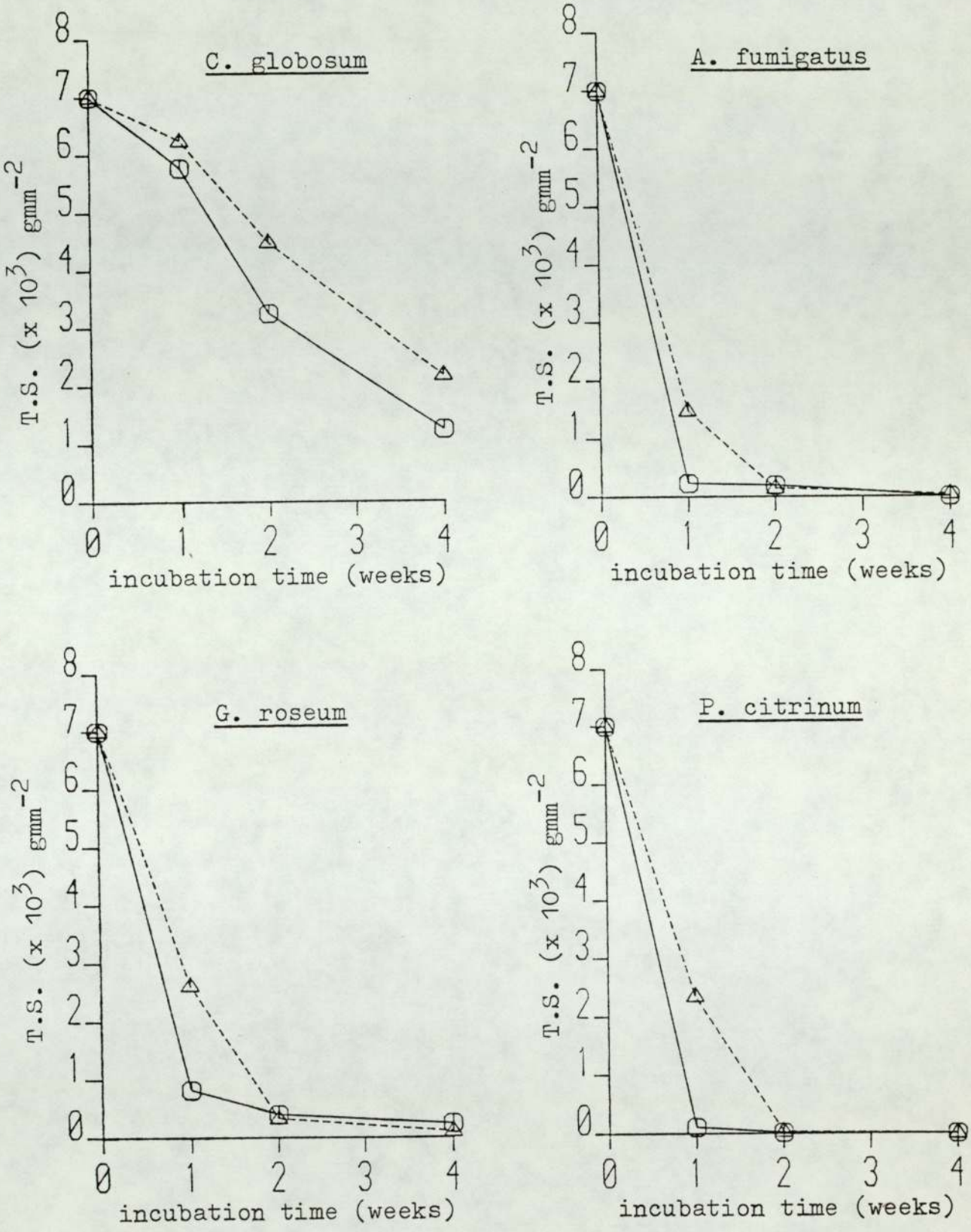


FIGURE 6.3

Tensile strengths (T.S.) of red medium polyester polyurethane when incubated with test fungi on MEA (△-△) and PLA (○-○) media

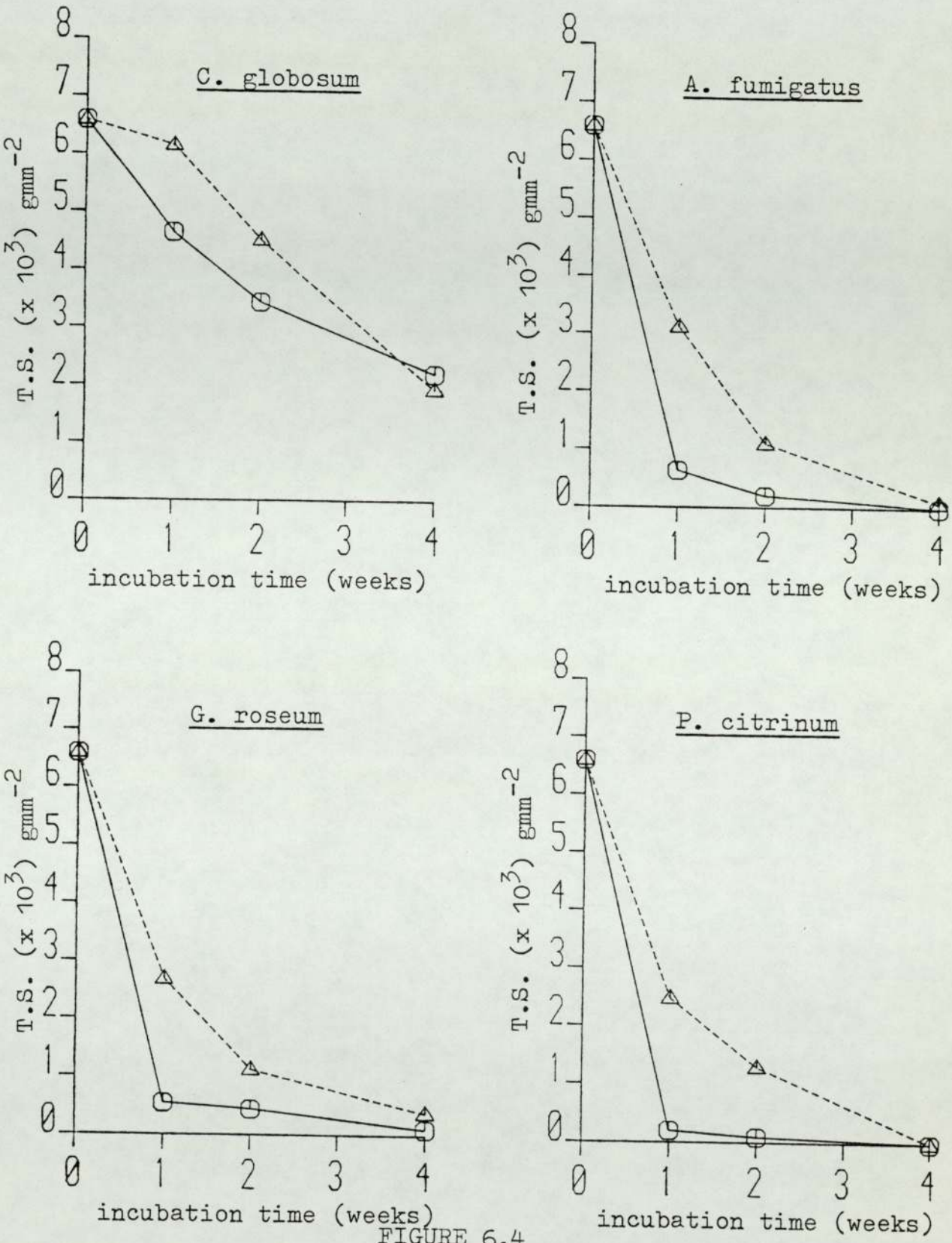


FIGURE 6.4

Tensile strengths (T.S.) of white hard polyester polyurethane when incubated with test fungi on MEA (△-△) and PLA (○-○) media

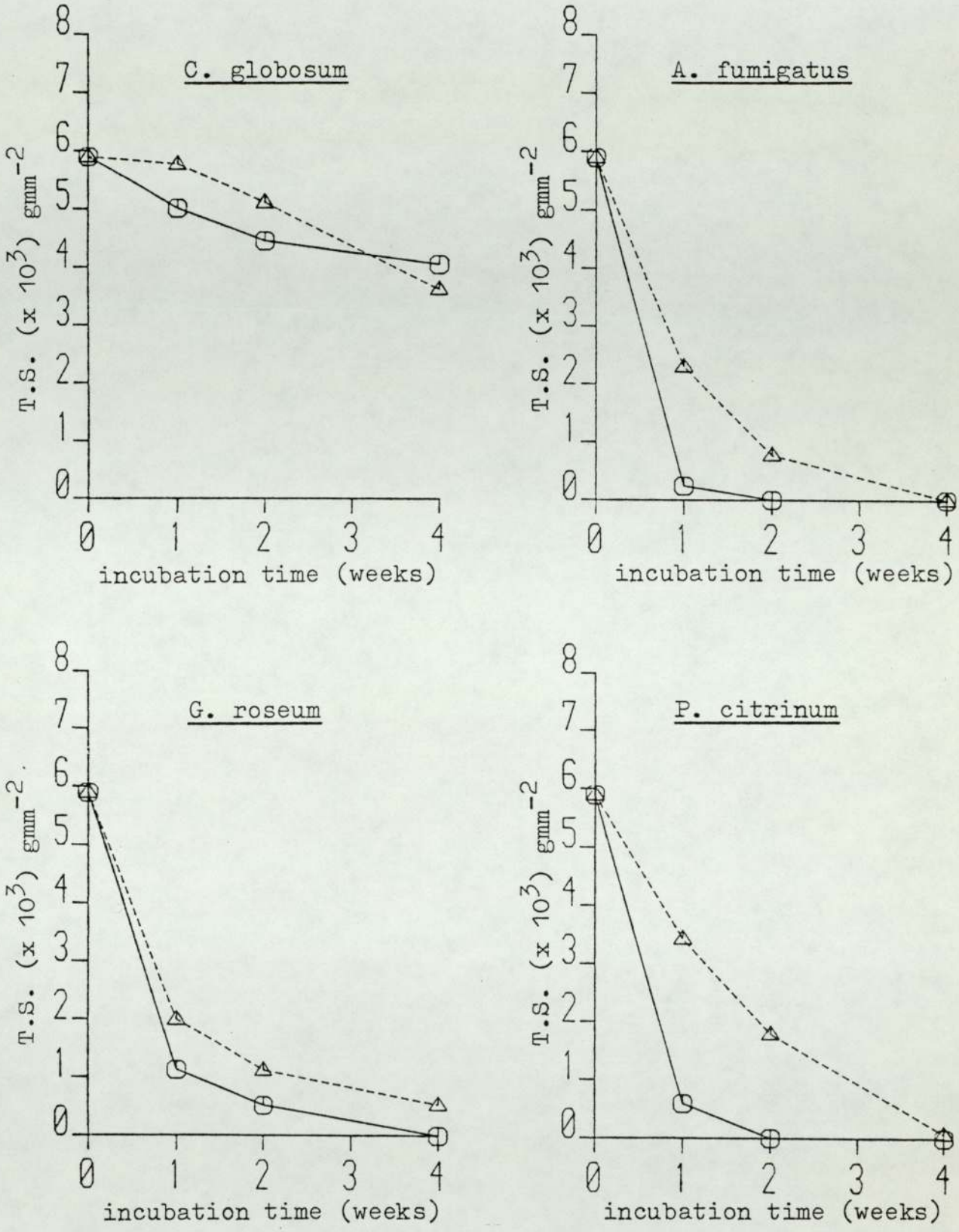


FIGURE 6.5

Tensile strengths (T.S.) of red hard polyester polyurethane when incubated with test fungi on MEA (△-△) and PLA (○-○) media

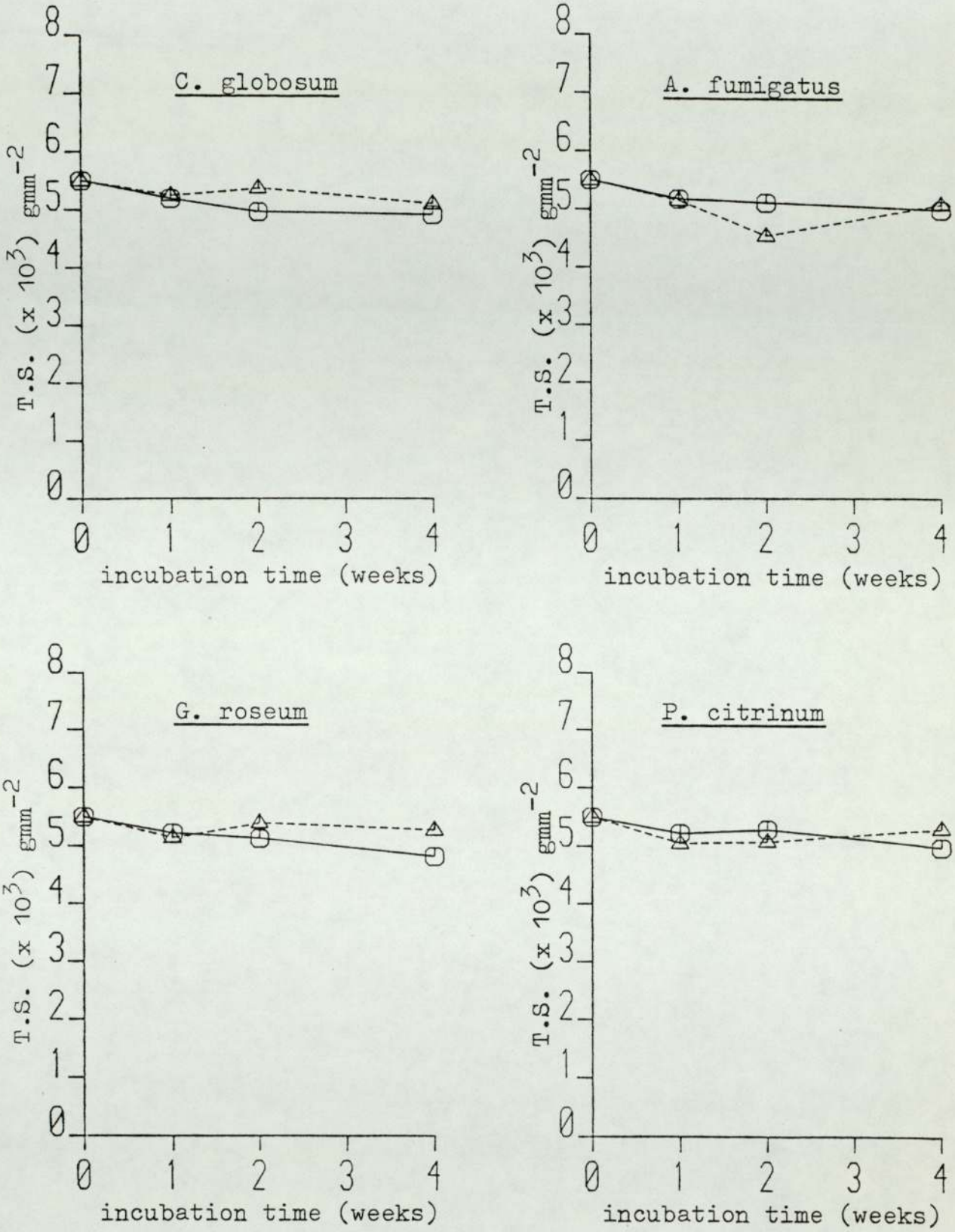


FIGURE 6.6

Tensile strengths (T.S.) of yellow hard polyether polyurethane when incubated with test fungi on MEA (△-△) and PLA (○-○) media

The tensile strengths of all four polyester type polyurethanes inoculated with test fungi, decreased rapidly with time of incubation. The effects of PLA and MEA media on the changes of tensile strengths with incubation time supported the observations made in Chapter 5 with changes in weight losses on the same media. In the early stages (1 to 2 weeks incubation) of the experiment, the tensile losses on PLA were significantly high than those observed on MEA, with all test fungi. But with longer times (after 4 weeks incubation) of incubation the gap between the tensile losses observed on PLA and MEA media reduced considerably and in some instances the order was even reversed.

In general, Penicillium citrinum, Aspergillus fumigatus and Gliocladium roseum exhibited almost the same magnitudes of tensile strength losses with all polyester polyurethane samples. These were significantly higher than the magnitude of tensile strength losses exhibited by Chaetomium globosum. Another observation made with Chaetomium globosum was the relative reduction of tensile strength losses with the increase in hardness of the test polyurethanes.

Figures 6.7 to 6.11 represent data obtained on percent ultimate elongation of yellow soft, red medium, white hard and red hard polyester type polyurethanes and yellow hard polyether type polyurethane elastomer respectively, with four fungi plotted versus the time of incubation. Each point represents the mean of six replicates.

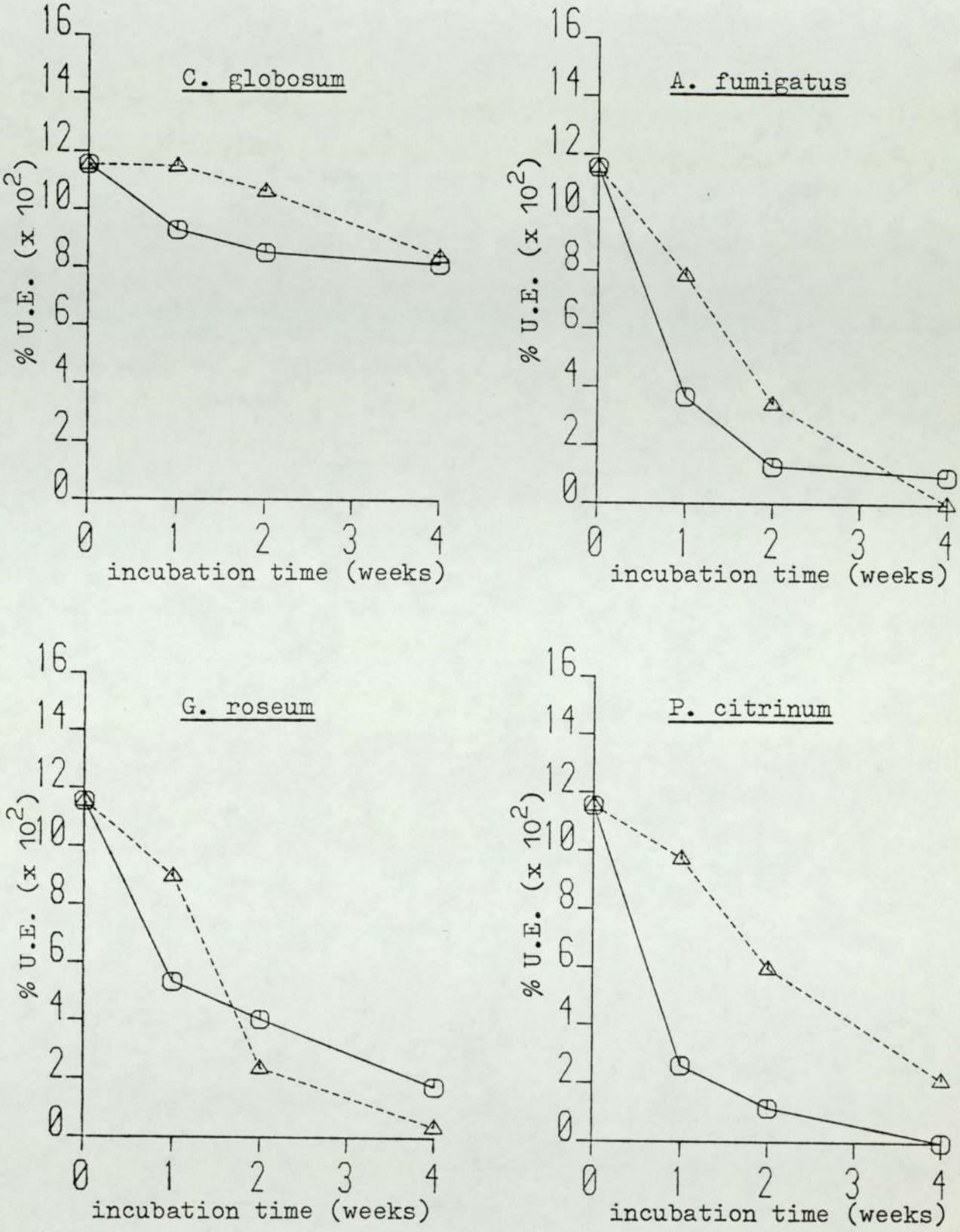


FIGURE 6.7

Percent ultimate elongations (% U.E.) of yellow soft polyester polyurethane when incubated with test fungi on MEA (△-△) and PLA (○-○) media

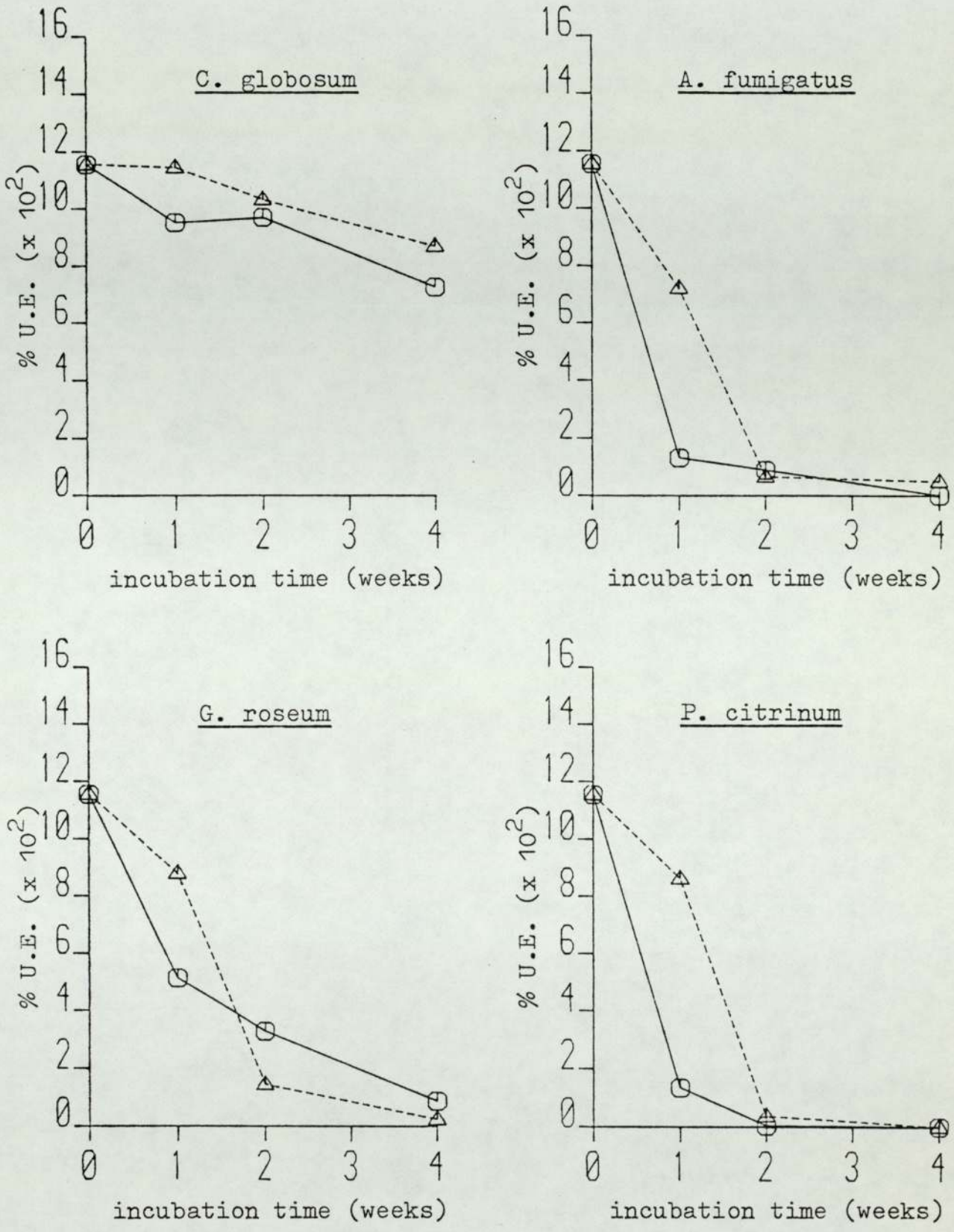


FIGURE 6.8

Percent ultimate elongations (% U.E.) of red medium polyester polyurethane when incubated with test fungi on MEA (△-△) and PLA (○-○) media

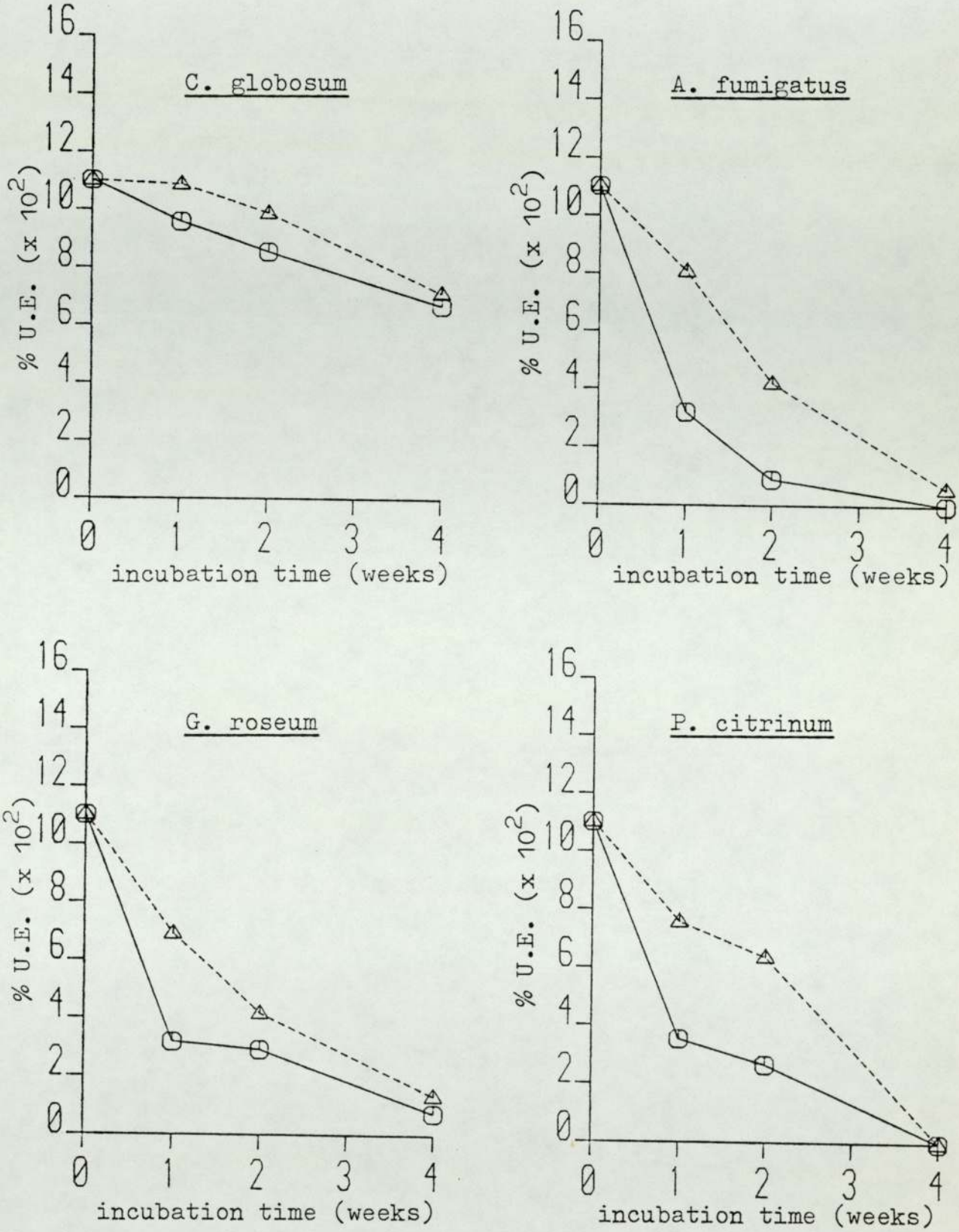


FIGURE 6.9

Percent ultimate elongations (% U.E.) of white hard polyester polyurethane when incubated with test fungi on MEA (△-△) and PLA (○-○) media

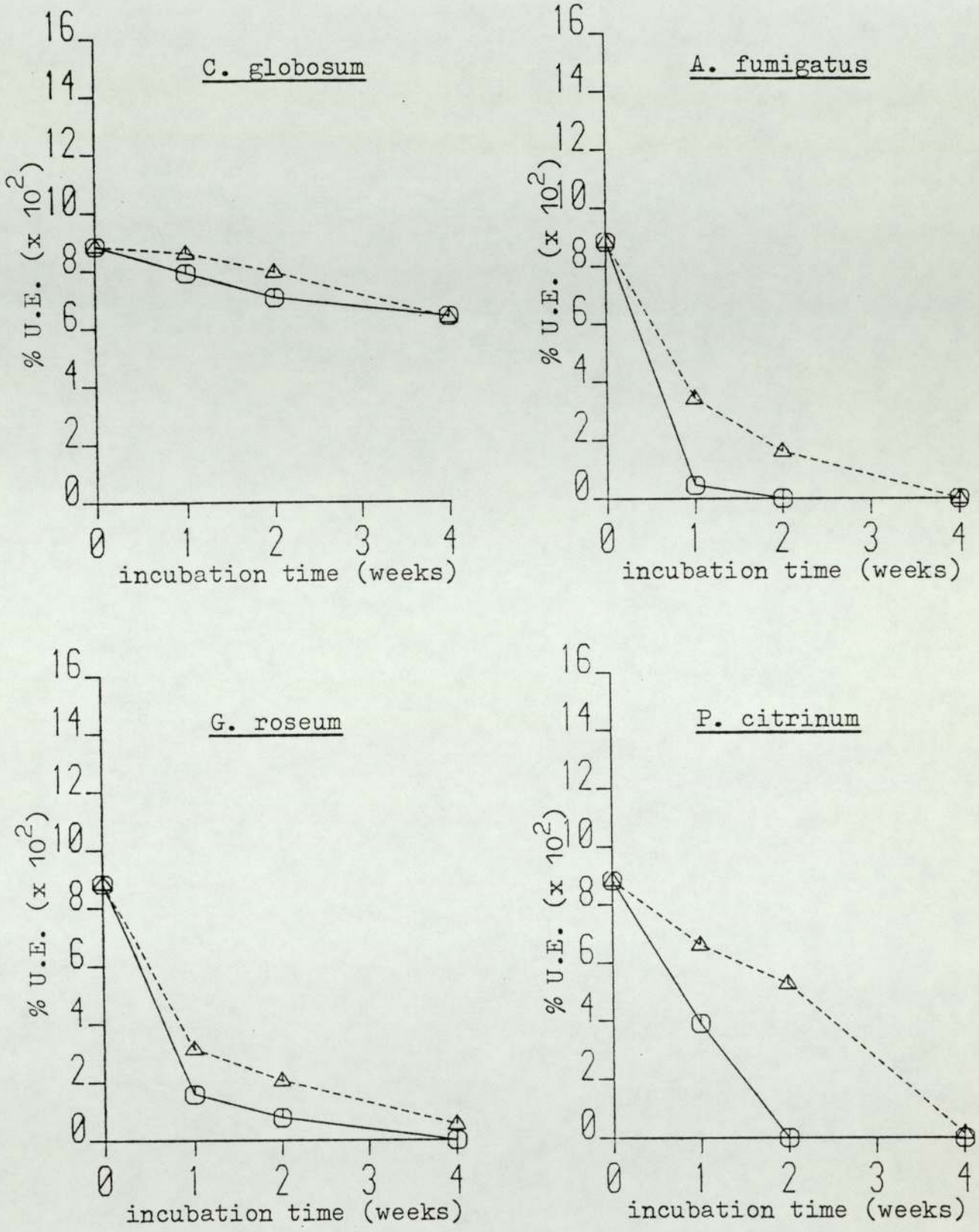


FIGURE 6.10

Percent ultimate elongations (% U.E.) of red hard polyester polyurethane when incubated with test fungi on MEA (△-△) and PLA (○-○) media

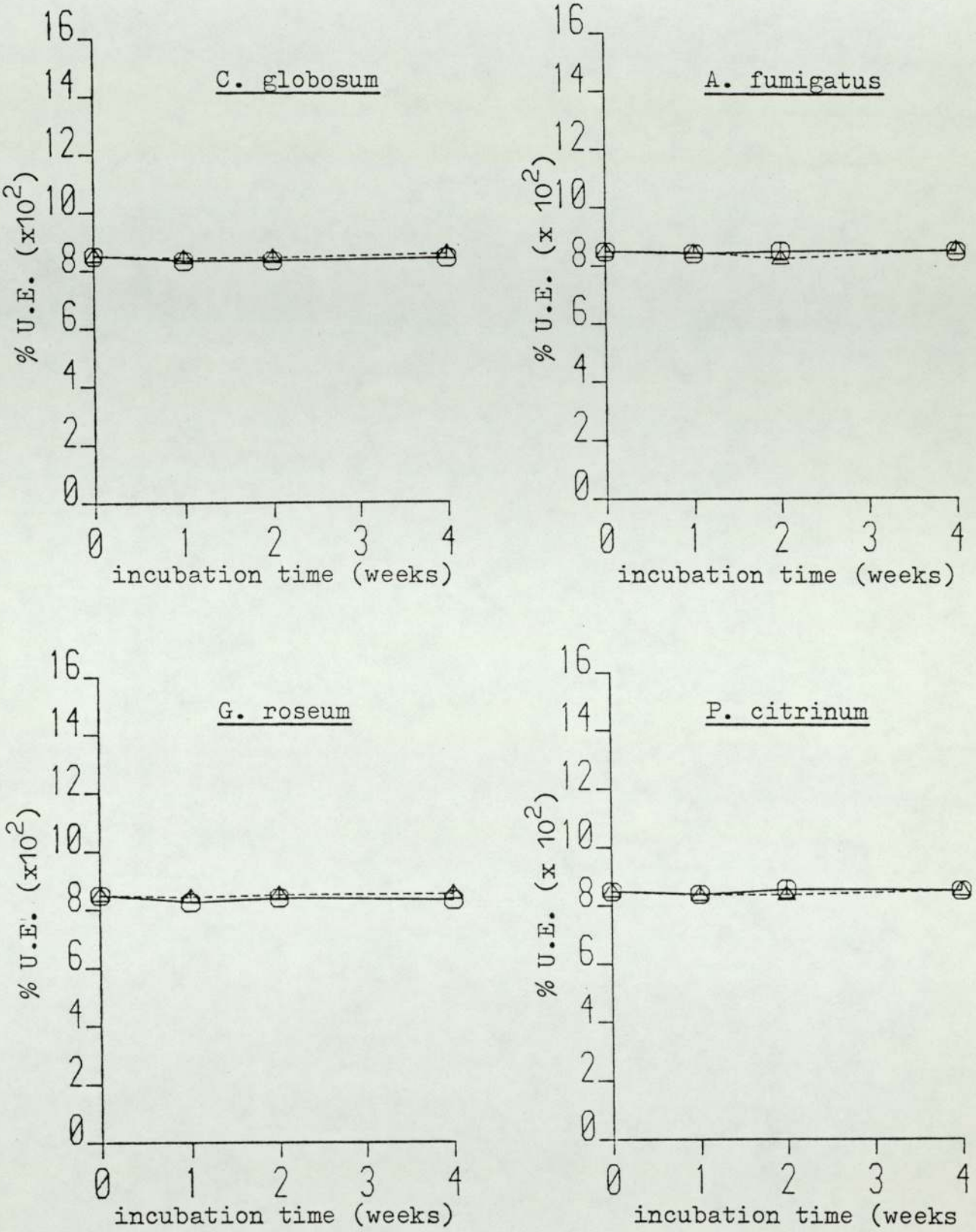


FIGURE 6.11

Percent ultimate elongations (% U.E.) of yellow hard polyether polyurethane when incubated with test fungi on MEA (△-△) and PLA (○-○) media

All polyester type polyurethanes exhibited (figures 6.7 - 6.10) varying degrees of percent ultimate elongations with different fungi, whereas, with polyether type the variations with different fungi remained insignificant (figure 6.11).

The percent ultimate elongations of all four polyester type polyurethanes decreased steadily but not as rapidly as tensile strengths with time of incubation. The effects of PLA and MEA media on percent ultimate elongations with time of incubation were very similar to those observed with tensile strength losses.

Although Penicillium citrinum, Aspergillus fumigatus and Gliocladium roseum showed slightly varying percent ultimate elongation results, they could be broadly categorized into one group. Chaetomium globosum which showed significantly lower changes in percent ultimate elongations with all four polyester type polyurethanes could be categorized into a separate group.

The means and standard deviations of changes in tensile strengths and changes in percent ultimate elongations were calculated and are presented in tables 6.5 to 6.9 and 6.10 to 6.14 respectively (appendix 4 and 5).

Tables 6.1 and 6.2 contain data obtained on tensile strengths and percent ultimate elongations respectively of white hard polyester polyurethane with four different fungi on different nutrient agar media after 10 days incubation.

TABLE 6.1

TENSILE STRENGTHS OF WHITE HARD POLYESTER POLYURETHANE WHEN INCUBATED WITH TEST FUNGI ON DIFFERENT NUTRIENT MEDIA

Medium	TENSILE STRENGTHS g/mm^2			
	G. roseum	C. globosum	P. citrinum	A. fumigatus
PLA	285 ± 205	3815 ± 255	114 ± 21	149 ± 70
PEA	674 ± 142	4320 ± 364	1344 ± 152	927 ± 265
UA	171 ± 44	5650 ± 296	4295 ± 320	565 ± 110
GA	327 ± 58	4304 ± 208	0 ± 0	44 ± 25
MEA	1110 ± 122	5064 ± 383	1016 ± 56	826 ± 111

N.B.

PEA = polyester agar

UA = urea agar

GA = gelatin agar

(appendices 1i, 1j, 1h)

TABLE 6.2

PERCENT ULTIMATE ELONGATIONS OF WHITE HARD POLYESTER POLYURETHANE WHEN INCUBATED WITH TEST FUNGI ON DIFFERENT NUTRIENT MEDIA

Medium	PERCENT ULTIMATE ELONGATIONS			
	G. roseum	C. globosum	P. citrinum	A. fumigatus
PLA	160.9 ± 111.0	965.2 ± 12.9	206.0 ± 107.4	80.4 ± 24.8
PEA	282.2 ± 88.9	1021.7 ± 20.0	609.6 ± 52.4	485.4 ± 108.7
UA	38.1 ± 16.0	1109.1 ± 20.1	1010.4 ± 14.4	306.2 ± 45.2
GA	176.4 ± 69.7	1099.3 ± 11.4	0.0 ± 0.0	17.6 ± 9.9
MEA	454.4 ± 43.6	1110.5 ± 19.8	524.9 ± 42.6	373.9 ± 34.4

N.B.

PEA = polyester agar

UA = urea agar

GA = gelatin agar

(appendices 1i, 1j, 1h)

As gelatin, polyester and urea supplements were added to the basal medium (PLA), the tensile strengths and percent ultimate elongations on these media were compared with the results on PLA. To make this comparison simple, changes of tensile strengths and percent ultimate elongations relative to changes observed on PLA are summarized in tables 6.3 and 6.4 respectively.

A significant increase was observed with both relative tensile strengths and relative percent ultimate elongations of all four test fungi on MEA and PEA media. On urea medium, except Gliocladium roseum which showed a decrease in relative tensile strength and percent ultimate elongation, the remainder showed an increase in both relative measurements. On gelatin agar, Penicillium citrinum and Aspergillus fumigatus showed a decrease in both relative measurements while Gliocladium roseum and Chaetomium globosum produced opposite results.

6.3 Discussion

% ultimate elongation and tensile strength are two measurable physico-mechanical properties of polymers. Both of these properties depend on the molecular structure and orientation of the polymer chains. Elongation of a polymer is a result of movement of polymer chains by stretching and sliding within the polymer. The extent of this movement depends on the length of the molecular chains. The strength of a polymer is a gross effect of molecular chains within a cross sectional area. Tensile strength measures the strength (load)

TABLE 6.3

RELATIVE VARIATION OF TENSILE STRENGTHS ON DIFFERENT MEDIA WITH RESPECT TO TENSILE STRENGTHS ON PLA MEDIA

Medium	RELATIVE VARIATION OF TENSILE STRENGTH (g/mm^2)			
	G. roseum	C. globosum	P. citrinum	A. fumigatus
PLA	0	0	0	0
PEA	+389	+ 505	+1230	+778
UA	-114	+1835	+4181	+416
GA	+ 42	+ 489	- 114	-105
MEA	+825	+1249	+ 902	+677

N.B.

(+) denotes an increase and

(-) denotes a decrease in relative tensile strengths.

TABLE 6.4

RELATIVE VARIATION OF % ULTIMATE ELONGATIONS ON DIFFERENT MEDIA WITH RESPECT TO % ULTIMATE ELONGATIONS ON PLA MEDIA

Medium	RELATIVE VARIATION IN % ULTIMATE ELONGATION			
	G. roseum	C. globosum	P. citrinum	A. fumigatus
PLA	0.0	0.0	0.0	0.0
PEA	+121.3	+ 56.5	+403.6	+405.0
UA	-122.8	+144.0	+804.4	+225.8
GA	+ 15.5	+134.1	-206.0	- 62.8
MEA	+293.5	+145.3	+318.9	+293.5

N.B.

(+) denotes an increase and

(-) denotes a decrease in relative percent ultimate elongations

necessary to extend the molecular chains within a cross-sectional area to breaking point. Therefore this property too depends on the chain length and flexibility of a polymer.

Both these properties become meaningful only when the changes in them due to some factor is compared. In this study the changes in tensile strength and percent ultimate elongation is caused by microbial deterioration. When a molecular chain within a polymer is attacked, the length of the molecule is reduced. This will result in a change in tensile strength and percent ultimate elongation. Initial breakage of polymer chains will affect tensile strengths more than percent ultimate elongations because tensile strengths depend on the gross strength of molecular chains within a unit cross-sectional area whereas ultimate elongations depend only on the longer chains within the polymer structure. This is clearly reflected in the results obtained in the present study.

Unlike the weight loss experiment described in Chapter 5, Penicillium citrinum showed relatively similar results in tensile strength losses and percent ultimate elongation changes to Aspergillus fumigatus and Gliocladium roseum. Chaetomium globosum, on the other hand, showed the least tensile strength losses and percent ultimate elongation changes as in the weight loss experiment.

Tensile strength and percent ultimate elongation results clearly indicate that during the process of microbial

degradation of polyurethanes there is severe reduction in the lengths of molecular chains and this is observed only with polyester type polyurethanes. The polyether type polyurethanes did not show relatively significant changes in tensile strengths and percent ultimate elongations indicating that its molecular structure is not affected by the microbial colonization.

The variations in tensile strengths and percent ultimate elongations of polyester polyurethanes on MEA and PLA with incubation time can be explained in the same way the weight losses were explained in Chapter 5.

The hypothesis (Chapter 5) of regular splitting and random splitting of molecular chains by 'polyurethane deteriorating enzymes' produced by fungi can be used to explain the tensile strength losses and percent ultimate elongation changes exhibited by different fungi. Tensile strength losses depend on the number of points at which the molecular chains are split in a unit cross-sectional area and not the amount of soluble units produced by splitting of molecular chains. Therefore, where changes in physico-mechanical properties are concerned, there is no difference in the type of attack reflected in the results. So the suggested 'regular splitting enzymes' in Penicillium citrinum and 'random splitting enzymes' in Aspergillus fumigatus and Gliocladium roseum (Chapter 5) will not show any significant variation in tensile strength losses and percentage ultimate elongation changes during degradation. This is exactly what is observed with the

tensile strength and percent ultimate elongation results. Therefore, this suggests that there are at least two significantly different enzyme systems involved in biodeterioration of polyurethanes. Chaetomium globosum, unlike others, only indicated that it produces relatively low concentrations of polyurethane degrading enzymes.

In the experiment on different nutrient media, the results were very similar to the results observed with weight losses. Therefore the explanations will be the same as the ones in Chapter 5.

The results on weight losses in Chapter 5 and on tensile strength losses and percentage ultimate elongations in this Chapter, have shown the changes in physical properties of polyurethanes during the process of biodeterioration by fungi. Therefore it is interesting to study the changes in chemical structures during the fungal deterioration process to obtain a complete picture of this complicated system and this is studied in the next Chapter.

CHAPTER SEVEN

INFRARED SPECTROSCOPY AND THIN LAYER CHROMATOGRAPHY STUDIES ON BIODETERIORATION OF POLYURETHANES

INFRARED SPECTROSCOPY AND THIN LAYER CHROMATOGRAPHY STUDIES ON BIODETERIORATION OF POLYURETHANES

The results of the changes in physical and/or mechanical properties of polyurethane elastomers, especially the polyester type, during fungal breakdown process, and the observations made with exoenzyme activities of test fungi, clearly indicated that biodeterioration of polyurethanes is caused mainly by chemical changes in the polymer structure. Therefore it was decided to investigate the possible chemical changes that occurred during the process of biodeterioration.

Filip (1978) studied the decomposition of polyurethane resilient foams by soil microorganisms using infrared spectroscopy. He showed that the two isocyanate shoulders at 2315 and 2120cm^{-1} almost completely disappeared and only a weak shoulder was left from the strong absorption band of the carbonyl frequency of ester and urethane groups at 1715cm^{-1} after 30 days incubation of polyester polyurethane foam with a mixed population of soil microorganisms. He used the KBr disc technique in this study.

Martens and Domsch (1981) studied the liberation of toxic aromatic amines in garbage landfill disposal conditions using ^{14}C-labelled polyurethane foams. They used radio-thin-layer chromatography to detect the aromatic amines liberated by hydrolysis of polyurethanes. Negligible amounts of amines were detected at ambient temperatures but at higher temperatures (50°C) aromatic amines were detected in

low concentrations. They suggested that at ambient temperatures, the amines produced are utilized by microorganisms but at higher temperatures the conditions are not suitable for growth of microorganisms and therefore accumulation of amines is possible.

In this study, both infrared spectroscopy and thin-layer chromatography were used to determine chemical changes that occurred during microbial degradation of polyester polyurethanes.

As the chemical structures of the polyurethane elastomers used in the present study were not disclosed by the manufacturer, a major problem was encountered when relating the products of biodeterioration to the original material. One test material, the white hard polyester polyurethane, was chosen for the study because it did not contain any additives. A complete chemical hydrolysis of the test polymer was performed to use hydrolysis products as reference materials for comparison with the products of microbial degradation.

7.1 Materials and methods

Chemical hydrolysis of white hard polyester polyurethane was initially carried out according to the method described by David and Staley (1969) with 40% sodium hydroxide solution, but, the hydrolysis was incomplete and neutralization was difficult. Therefore, instead of using 40% NaOH solution, a 10% NaOH solution was used and refluxing was

continued for 3 hours. The polyurethane showed complete hydrolysis, but, the amine component was very unstable in basic solution and oxidized rapidly turning the solution purple. Another problem encountered was the large quantities of NaCl formed in the neutralization process. As the base hydrolysis created many problems, acid hydrolysis with 3N HCl solution was examined. The rate of hydrolysis was much slower than with NaOH, and complete hydrolysis was attained after 5 days of refluxing. The amine portion was quite stable in acid and showed no oxidation. To avoid salt formation due to neutralization, the hydrolysed extract was evaporated in a fume-cupboard at room temperature. Then the precipitated solids were dissolved in dilute HCl solution (0.01N HCl) and a thin layer chromatogram was run on Silica Gel 60 plates using the top layer of n-butanol : acetic acid : water (4 : 1 : 5) as the developing solvent system (Gillio-Tos et al., 1964). The amine was detected under UV light (Gillio-Tos et al., 1964) as a bright blue fluorescent spot. It also turned purple after some time due to oxidation. The acid was detected by spraying with bromocresol green indicator (Braun et al., 1962). The acid spot turned yellow in a blue background. The alcohol portion was not isolated but the amine and acid were enough as reference compounds.

The polyurethane sheets supplied by the manufacturer were too thick for spectral analysis. These were impossible to dissolve in any standard organic solvent and therefore no quantitative studies of chemical changes during bio-deterioration were possible. The alternative was to compare

the chemical changes using the KBr disc method with the ground polymer. A suitable method of grinding the undegraded elastomer could not be found, so the studies were limited to the undegraded polymer and the completely embrittled polymer. Penicillium citrinum was used as the test fungus because it exhibited the maximum weight loss and also embrittled the test material completely.

A Perkin-Elmer 599 B infrared spectrophotometer was used to analyse the samples. A chart recorder automatically plotted a graphical representation of the transmittance over the range of 4000 to 650cm^{-1}.

To study the chemical changes due to fungal deterioration, 4g of the surface-sterilized polyester polyurethane pieces (2.0cm x 0.5cm) were spread evenly on the surface of 50ml of PLA medium in a glass petri dish (diameter 15cm) in duplicate. These were inoculated with 1ml each of spore suspension of Penicillium citrinum and incubated at 30°C for 6 weeks, sealed in polythene bags to avoid desiccation. Then the surface mycelium was very carefully scraped off and the degraded pieces were washed in several changes of water, ground with a mortar and pestel, made in an aqueous suspension in 100ml of distilled water and filtered through a sintered glass funnel no. 1 under suction. The precipitate was washed in distilled water and then dried in a vacuum desiccator for 48 hours.

The undegraded polymer was scraped using a very fine file and the scrapings were used to record the infrared

spectrum. The KBr disc technique was used to record the spectra of both degraded and undegraded samples. As a reference, the infrared spectrum of the acid fraction of the chemically hydrolysed polymer was recorded.

Further investigations on possible chemical changes during biodeterioration of polyurethanes were carried out using another type of industrial polyester polyurethane elastomer, which was obtained as a colourless film of about 0.02cm thick. This was too thick for spectral studies, but, it could be dissolved in dimethyl formamide. During recasting the polymer precipitated with the slightest absorption of moisture. To overcome this problem a positive pressure dry air cabinet was designed (figure 7.1). Using this system, films up to 0.002cm of uniform thickness were prepared which gave reasonably good infrared spectographs. In this study Gliocladium roseum was used as the test fungus, because it particularly showed extensive degradation of this polymer.

The film was placed on MEA plates and inoculated with 0.5ml spore suspension of Gliocladium roseum, and was incubated at 30°C for 2 months, sealed in polythene bags to avoid desiccation. Then the fungus was very carefully washed off by rubbing between the fingers and dried in a vacuum desiccator for 48 hours. Infrared spectra of the film were recorded before and after the experiment.

7.2 Results

The undegraded white hard polyester polyurethane showed

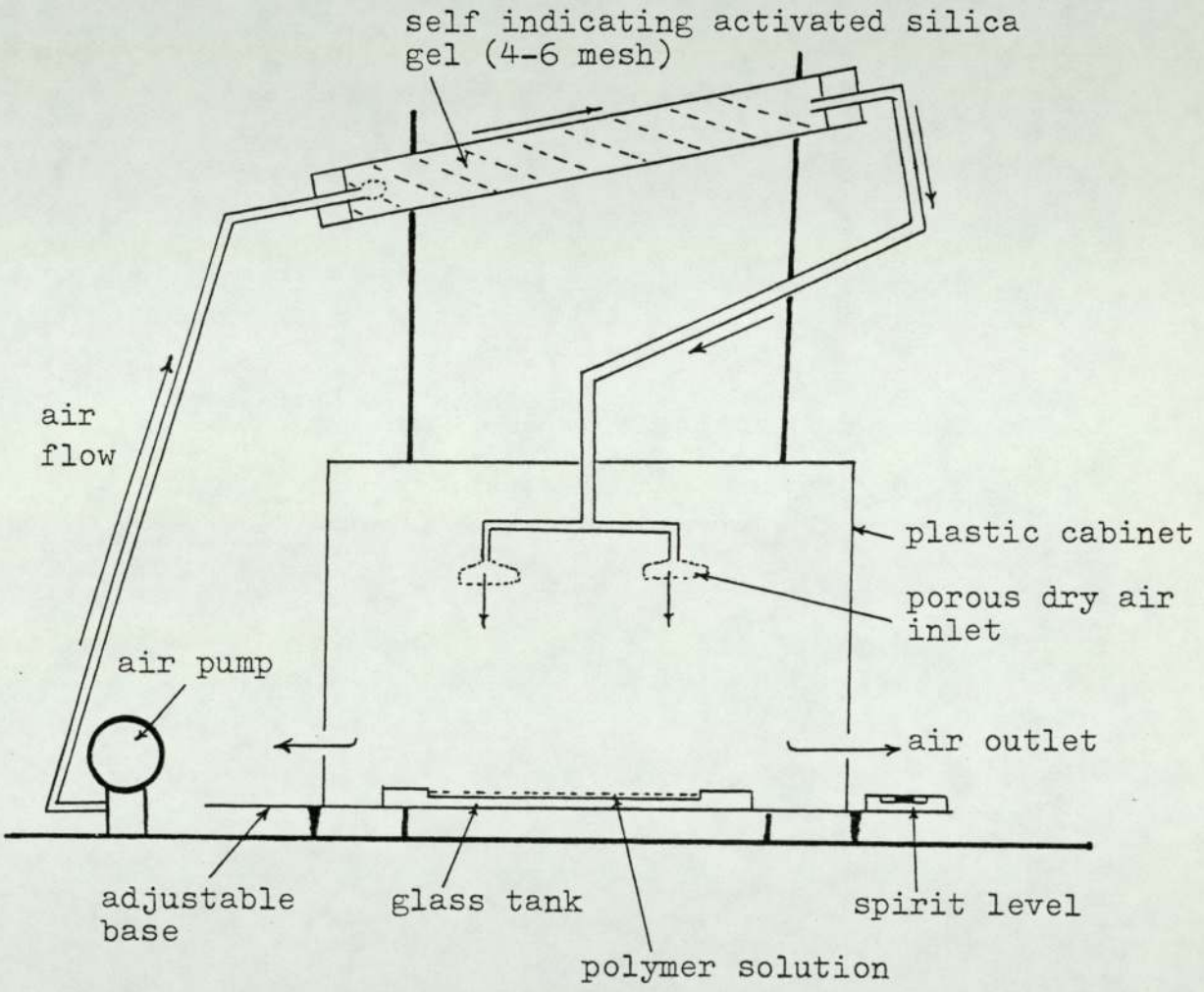


FIGURE 7.1

Positive pressure, dry air cabinet, to prepare polyurethane films for spectral studies

a strong carbonyl absorption at 1735cm^{-1} and no absorption at 1685cm^{-1}. The acid fraction gave strong absorption at 1685cm^{-1} and no peak at 1735cm^{-1}. The degraded polyurethane showed absorption at 1735cm^{-1} and in addition a strong peak at 1685cm^{-1}. Both peaks overlapped but were significantly prominent at these two wave numbers. These observations are clearly represented in figure 7.2. Another observation made with the undegraded and degraded polyester polyurethane samples were the relative changes in N-H stretch (3350cm^{-1}) and C-H stretch (2950cm^{-1}) (figure 7.3). In the undegraded polymer the peak due to C-H stretch was relatively bigger than the N-H stretch. In the degraded polymer the C-H stretch peak was significantly smaller than the peak due to N-H stretch. This indicated a relative decrease in C-H stretch peak during biodeterioration.

An accidental observation led to an interesting discovery with one of the two plates inoculated with Penicillium citrinum used for this experiment. As one plate provided enough degraded substance for spectral studies, the other plate was left on a laboratory table for two weeks without sealing in a polythene bag. Within this period the medium almost dried and during this process a white chemical, probably an organic compound, had crystallized at several spots just below the test pieces within the agar medium. These crystals were carefully removed and were compared with the chemically hydrolysed products chromatographically using the previously described thin layer method. This compound looked chromatographically almost pure and showed a yellow spot parallel to the acid fraction when sprayed with

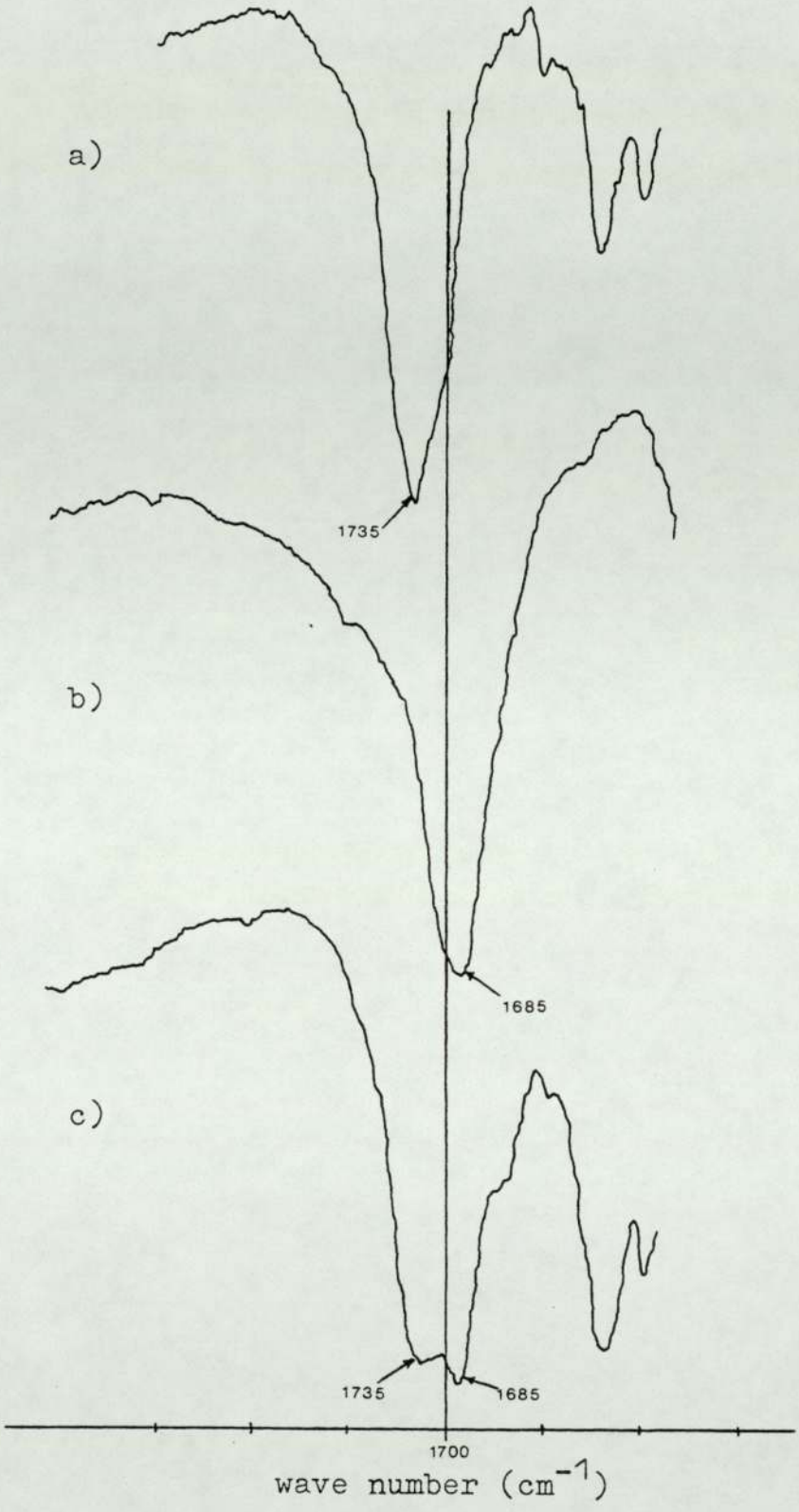


FIGURE 7.2

Infrared spectra of carbonyl peaks of a) white hard polyester polyurethane b) acid fraction of the polyester polyurethane and c) degraded polyester polyurethane

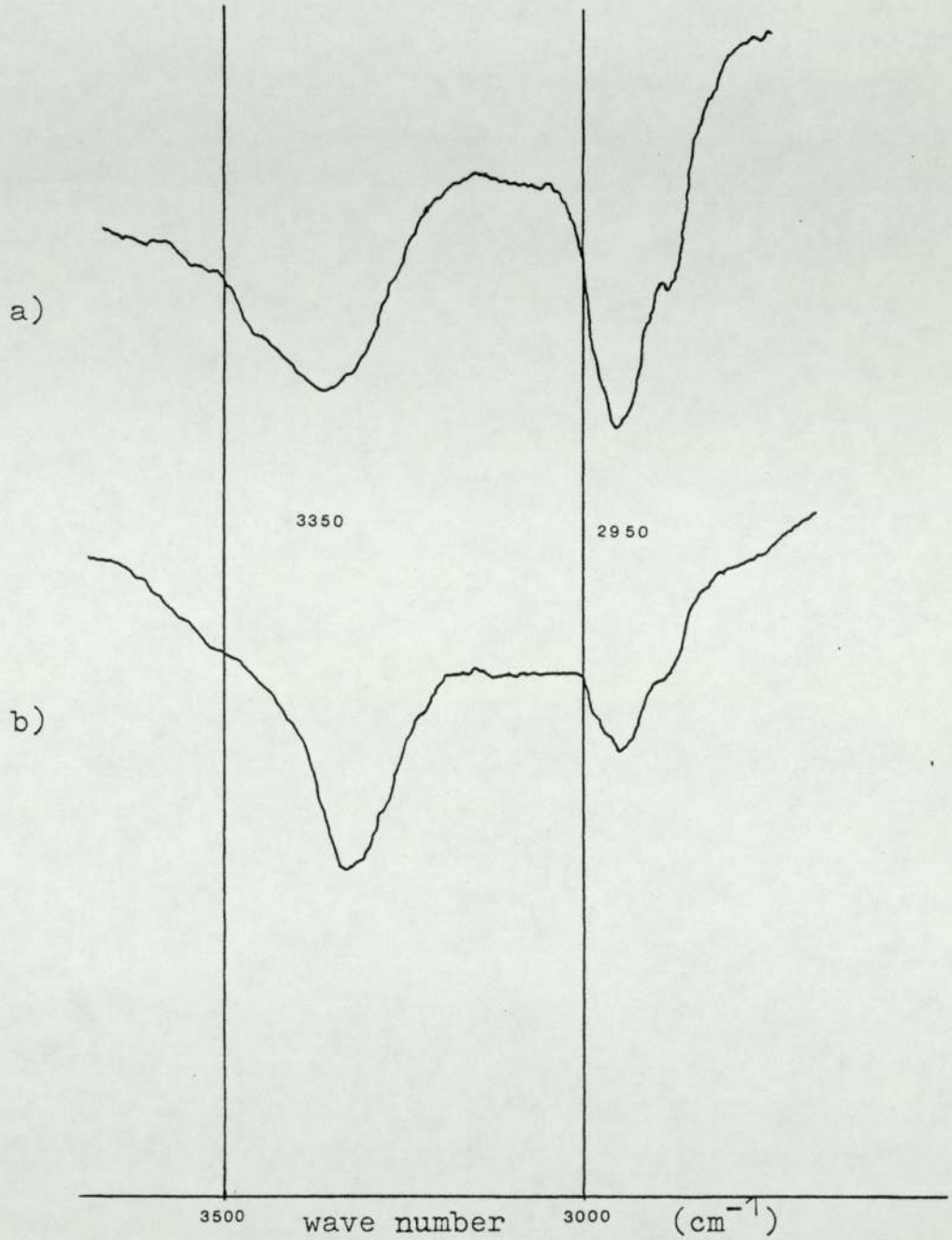


FIGURE 7.3

Infrared spectra of N-H stretch (3350 cm^{-1}) and C-H stretch (2950 cm^{-1}) of a) undegraded and b) degraded white hard polyester polyurethane

bromocresol green indicator.

Very minute differences in the spectra of degraded and nondegraded polymer films were observed but these were not considered adequate to be able to distinguish chemical modification which may have occurred. The small differences may have been due to operating differences when carrying out measurements since the instrument was in continual use and it was necessary to readjust it prior to each measurement.

7.3 Discussion

Hydrolysis of polyester type polyurethanes gives rise to diacids, diamines, dialcohols and substituted ureas (David and Staley, 1969). Diacids and dialcohols are products of polyester hydrolysis and diamines arise from the hydrolysis of urethane units. Substituted ureas represent the branching points. Therefore the presence of diacids or dialcohols as byproducts of biodeterioration indicate hydrolysis of the polyester region of the polymer molecules. Presence of diamines or substituted ureas means breakdown of the urethane bonds.

In the biodeterioration experiment with Penicillium citrinum, the presence of a free acid fraction in the medium indicated hydrolysis of the polyester region probably by esterases. Accumulation of acid showed that the rate of utilization by the fungus was slower than the rate of breakdown of the polyester region of the polyurethane into free acids.

The appearance of a carbonyl peak at 1685cm^{-1} during degradation of polyester polyurethane, which corresponded to the carbonyl peak shown by the free acid indicated a build up of free carboxyl groups ($-C\overset{O}{\underset{OH}{<}}$) during degradation. This supports the explanation of a hydrolysis of the polyester region of the polyurethane during biodeterioration. The relative reduction of the C-H stretch peak too indicated a loss of $-CH_2-$ groups from the polyurethane. The diacid and most probably the alcohols contribute to the major portion of $-CH_2-$ groups of polyurethane. Therefore, the relative reduction of the intensity of C-H stretch during degradation indicate a loss of diacid and dialcohol groups from the polymer.

The degradation of polyester polyurethane film by Gliocladium roseum, although visually very significant, did not show significant changes in infrared spectra. This may indicate that the effects of deterioration of the polymer are very small and subtle with the major gross polymeric structure being relatively unchanged and so masking these very small changes.

CHAPTER EIGHT

DISCUSSION, CONCLUSIONS AND PROPOSALS FOR FUTURE WORK

DISCUSSION, CONCLUSIONS AND PROPOSALS FOR FUTURE WORK

The great preponderance of investigative work done on biodeterioration of polyurethanes during the late 1960s and early 1970s and even to this day, is largely empirical. Very little is understood about the actual mechanism involved in fungal deterioration of polyurethanes and very few research groups are currently investigating this phase of the problem.

Early investigators concentrated their attention mainly on the polyurethanes as materials in service and used fungi merely as indicators of the susceptibility or resistance of these polymers. They thought that this problem could be simply solved by incorporating a fungicide in the formulation. However, incompatibility of the fungicides with the formulations appeared to be more of a problem with polyurethanes than with other polymeric systems. Each polyurethane system had to be evaluated individually to make certain that the fungicide employed was active and compatible under use conditions and it was a quite difficult task to be achieved.

From the mid-1970s there was a steadily increasing demand for polyurethanes and most probably due to this, the publications on susceptibilities of these products to microorganisms disappeared suddenly. This created the impression that the susceptibility of polyurethanes to microbial attack was permenantly solved. This did not prevent the microorganisms from continuing their attack on polyurethanes.

Many polyurethane based products failed much earlier than their expected service times and many consumers started complaining about these products (Seal, 1979). These reports initiated the study undertaken and presented in the previous seven chapters.

So, with the view of finding a permanent solution to this problem, a foundation was laid in this study to understand the mechanism of polyurethane deterioration by understanding the mechanisms by which fungi deteriorate polyurethanes and the conditions which encourage this to take place.

At the beginning of this project, due to limited facilities available, the idea of synthesizing polyurethanes was dropped and it was decided to use commercially available polyurethanes for this study. The early response from the manufacturers was rather poor and the earlier work was limited only to a polyester type poromeric polyurethane used in the shoe industry, which was used in the initial screening experiments. Even with these limitations, five fungi, namely: Gliocladium roseum, Chaetomium globosum, Penicillium citrinum, Aspergillus fumigatus and Nigrospora spherica imposed deteriogenic effects on the material and these were chosen for further studies.

Three of the fungi used in this study as test organisms, namely: Gliocladium roseum, Aspergillus fumigatus and Nigrospora spherica have not been reported as polyurethane deteriogens in the past literature. This clearly emphasizes the importance of isolation and identification of all fungal species which show positive deteriogenic effects on polyurethanes. This

enables the committees which consider standards for biological testing of plastics to review their list of test species when updating test procedures.

The most important fact in microbial deterioration of polyurethanes which most plastic engineers still do not fully understand is the failure of a particular polyurethane formulation at ambient temperatures when it proved to be almost completely resistant to ultra violet, oxidation, ozone, irradiation and even hydrolysis. This type of problem should have been clarified and explained long ago but unfortunately it has not been properly achieved still and the blame must fall on biologists and biochemists for not doing enough research on this aspect.

The importance of the role of enzymes for survival of life on this planet and their versatility is still a subject limited only to researchers and students in biology and biochemistry. It is true that the knowledge on this subject has been gained in the relatively recent past, but, now the time has come to convey this message in proper scientific terms to researchers and students in non-biological fields who contribute to the production of the major portion of materials used for the continuation and enhancement of life on this planet.

When the role of microorganisms is considered, their ability to survive in extreme environments and utilize almost any substrate with which they come into contact,

mainly by producing enzymes is quite fascinating and still not properly understood. Their ability to sense a particular substrate and to induce production of suitable enzymes is something that needs explaining, if a problem like microbial deterioration of polyurethanes has to be permanently solved. Therefore in this study, with the existing knowledge an attempt was made to find a suitable path in solving this problem. Protease, esterase and urease enzymes were selected for study after initially considering the structure of polyurethanes. This did not mean that these enzymes were solely responsible for polyurethane deterioration, and existence of a separate polyurethane degrading enzyme system or 'polyurethanase' is quite possible.

Comparison of enzyme activities, among fungi and also with degradation results were made to study the relationship between enzymes and biodeterioration. The relatively low initial rates of degradation on MEA with respect to PLA showed that abundant sugars in MEA caused catabolite repression of the 'polyurethanases'. This indicated that 'polyurethanases' are inducible enzymes (Stadtman, 1970). The relative increase in degradation rates with all fungi when gelatin was used in the medium indicated that gelatin induced production of 'polyurethanases'. The opposing results observed when polyester was used in the medium cannot be explained as catabolite repression and this may be due to competitive inhibition of 'polyurethanases' by the polyester present in the medium. This shows that polyurethanases can also hydrolyse the polyesters and thus the esterases could possibly be potential 'polyurethanases', or a fraction of them. This

is further supported by the facts that polyester type polyurethanes were more susceptible than polyether type polyurethanes and also by the presence of free acid in the medium after deterioration of white hard polyester polyurethane by Penicillium citrinum. When the enzyme activities of test fungi were compared with their abilities to degrade polyurethanes, only esterase activities correlated well with the biodeterioration rates. The build up of free carboxylic groups during the biodeterioration process, which was indicated by the infrared spectral analysis and also the relative reduction of $-CH_2-$ groups too supported the breakdown of polyester region of the polyurethane. All these observations indicate that there is a close relationship between the 'polyester hydrolysing' enzymes and 'polyurethane hydrolysing' enzymes. The properties of 'polyester hydrolysing' enzymes showed some variation among different species which was reflected in the action of 'polyurethane hydrolysing' enzymes. The clearance patterns of polyester medium, the weight loss and mechanical testing results indicated that there are at least two different types of 'polyurethanases' differing in their modes of attack. It is quite important to note that a generalized term like esterases does not reflect the real importance or complexity of the induced enzyme systems that are responsible for polyurethane deterioration.

The effects of 'polyurethanases' are reflected in the changes of physico-mechanical properties and weights of the polymer. To study these effects, percent ultimate elongations and weight losses have been calculated at 50% tensile strength

losses for ease of comparison and they are summarized in tables 8.1 to 8.4 for yellow soft, red medium, white hard and red hard polyester type polyurethanes respectively.

The time taken to attain 50% tensile strength loss shows that Gliocladium roseum, Penicillium citrinum and Aspergillus fumigatus achieved this at early stages of the experiment. This is quite important in explaining these observations. On PLA medium there were limited nutrients and the fungi were induced to produce 'polyurethanases' from the start. But, on MEA medium there was catabolite repression due to abundant sugars and the production of induced 'polyurethanases' were delayed. At the same time there was heavy production of fungal biomass due to abundant supply of nutrients. Once the sugars were utilized, the effects of catabolite repression disappeared and the fungi started producing large quantities of 'polyurethanases' with respect to relatively low amounts of enzymes produced by the relatively low biomass on PLA medium. Therefore with a relatively low concentration of enzymes gradually but steadily produced on PLA medium, the fungi will take longer time to achieve 50% tensile loss. At the same time, it will allow the enzymes to penetrate deep into the material causing widespread breaking of the polymer chains. But, on MEA medium due to relatively large quantities of enzymes produced at the same time, a shorter time period will be necessary to achieve 50% tensile strength loss and this means less penetration and lesser number of long chains attacked and relatively few chains completely broken.

TABLE 8.1

PERCENT ULTIMATE ELONGATIONS, PERCENT WEIGHT LOSSES AND TIME TAKEN FOR TEST FUNGI TO ATTAIN 50% TENSILE STRENGTH LOSS OF YELLOW SOFT POLYESTER TYPE POLYURETHANE ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	TIME TAKEN (WEEKS)	% ULTIMATE ELONGATION	% WEIGHT LOSS
G. roseum	MEA	0.79	950	2.2
	PLA	0.56	795	4.2
C. globosum	MEA	2.80	970	9.0
	PLA	0.80	970	4.4
P. citrinum	MEA	0.87	1000	14.2
	PLA	0.52	675	19.0
A. fumigatus	MEA	0.65	905	1.8
	PLA	0.54	708	2.5

TABLE 8.2

PERCENT ULTIMATE ELONGATIONS, PERCENT WEIGHT LOSSES AND TIME TAKEN FOR TEST FUNGI TO ATTAIN 50% TENSILE STRENGTH LOSS OF RED MEDIUM POLYESTER TYPE POLYURETHANE ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	TIME TAKEN (WEEKS)	% ULTIMATE ELONGATION	% WEIGHT LOSS
G. roseum	MEA	0.80	935	2.0
	PLA	0.54	800	4.1
C. globosum	MEA	2.84	960	8.9
	PLA	1.87	970	9.6
P. citrinum	MEA	0.75	940	9.1
	PLA	0.50	640	17.0
A. fumigatus	MEA	0.64	870	1.5
	PLA	0.54	590	3.0

TABLE 8.3

PERCENT ULTIMATE ELONGATIONS, PERCENT WEIGHT LOSSES AND TIME TAKEN FOR TEST FUNGI TO ATTAIN 50% TENSILE STRENGTH LOSS OF WHITE HARD POLYESTER POLYURETHANE ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	TIME TAKEN (WEEKS)	% ULTIMATE ELONGATION	% WEIGHT LOSS
G. roseum	MEA	0.84	750	2.1
	PLA	0.54	670	8.9
C. globosum	MEA	2.94	855	11.7
	PLA	2.22	825	7.8
P. citrinum	MEA	0.80	805	12.0
	PLA	0.52	700	19.8
A. fumigatus	MEA	0.94	815	4.0
	PLA	0.54	680	7.8

TABLE 8.4

PERCENT ULTIMATE ELONGATIONS, PERCENT WEIGHT LOSSES AND TIME TAKEN FOR TEST FUNGI TO ATTAIN 50% TENSILE STRENGTH LOSS OF RED HARD POLYESTER POLYURETHANE ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	TIME TAKEN (WEEKS)	% ULTIMATE ELONGATION	% WEIGHT LOSS
G. roseum	MEA	0.75	445	1.0
	PLA	0.60	430	3.3
C. globosum	MEA	-	-	-
	PLA	-	-	-
P. citrinum	MEA	1.30	610	13.7
	PLA	0.55	600	14.7
A. fumigatus	MEA	0.82	440	1.4
	PLA	0.52	435	4.7

When percent ultimate elongation results at 50% tensile strength loss is considered, the percentage ultimate elongations on PLA medium was considerably less than on MEA medium. As the percent ultimate elongation depends on the number of longer chains in the polymer the above arguments justify these results.

The same arguments can be used to explain the variations of percent weight losses on PLA and MEA media, in that there is more time for enzymes to produce more water soluble smaller molecular fractions on PLA medium than on MEA medium. The total time taken to achieve 50% tensile strength loss on MEA medium is greater than on PLA medium because of the lag period due to catabolite repression, when no 'polyurethanases' were produced.

Chaetomium globosum, on the other hand, took a longer time to achieve 50% tensile loss. By this time the initial advantage on PLA medium was lost and even overtaken by the relatively large quantities of enzymes produced on MEA after catabolite repression. This is the reason why percent ultimate elongations and percent weight losses at 50% tensile strength loss are similar or even greater on MEA.

When different fungi are compared, Gliocladium roseum and Aspergillus fumigatus showed similarities in percentage weight losses and percent ultimate elongations at 50% tensile strength losses. Penicillium citrinum too showed similar percent ultimate elongations but the weight losses were considerably higher than that of any other test fungi.

This could only be explained by considering the modes of attack of the enzymes produced by these fungi and also assuming that the polyester unit is the most susceptible fraction of the polymer to biodeterioration. Work on esterases suggested that Penicillium citrinum produced enzymes which hydrolyse polyesters regularly into water-soluble acid and alcohol groups whereas Gliocladium roseum and Aspergillus fumigatus hydrolyse the polyester randomly sometimes leaving insoluble fractions behind. If the structures of the polyurethane molecules are considered, the length of an acid or an alcohol unit within a polyurethane molecule is relatively very small. So once a polymer is broken at a point, loss of a few acid and alcohol units from either side will not significantly change the length of the two molecular pieces. Therefore this will not affect a significant variation in percent ultimate elongation. But, on the other hand, it will considerably increase the soluble fraction of the polymer, thus increasing percent weight loss of the polymer.

Due to limited time period, the separation and identification of all the byproducts of biodeterioration process could not be achieved completely. But the detection of the acid, used in the manufacture of polyurethane, in the medium, indicated that hydrolysis of the polyester region is one of the paths that fungi use to degrade polyurethanes. The build up of free carboxyl groups and relative reduction of C-H stretch during biodeterioration process indicated by infrared spectra analysis too supported the above explanation. These two changes in infrared spectra can be used as a quantitative measurement of biodeterioration of polyester polyurethanes

in future studies.

The present study indicates the complexity of biodeterioration process of polyurethanes, mainly the polyester type. Because of this reason it is impossible to obtain a complete idea of this process using a single test method. It is clearly shown in this study that use of test methods such as weight losses or changes in physico-mechanical properties as the sole test method to determine the effects of biodeterioration may produce results which could be misleading. Therefore, it is necessary to compare the results of several test methods which cover different parameters when a complex system like this is studied.

Finally, it can be concluded that polyester type polyurethanes are significantly more susceptible to fungi than the polyether type polyurethanes, which are almost completely resistant to fungal deterioration. The most susceptible region of polyester polyurethanes is the polyester unit. Fungi use inducible enzymes to achieve this. The splitting of molecular chains takes place probably via two paths i.e. random splitting and regular splitting. Gliocladium roseum and Aspergillus fumigatus, the two fungi introduced to the field of biodeterioration of polyurethanes, were quite efficient in causing breakdown of polyester polyurethanes. Penicillium citrinum too was highly effective in breaking down the polyester polyurethane elastomers. The least effective of the test fungi was Chaetomium globosum.

The results obtained during this project indicate that

more work has to be done for a complete understanding of the process of biodeterioration of polyurethanes. Therefore the following areas of research can be recommended for future work in the light of the observations made during this research programme:-

a. Select fungal species for specific enzyme production.

b. Extract cell free enzymes and purify.

c. Use polyurethanes of known compositions.

d. Determine breakdown pattern of polyurethanes using purified enzymes.

e. Determine factors affecting this breakdown.

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APPENDICES

Appendix 1

Media used in experimental work

Appendix 2

Polyurethanes used in experimental work

Appendix 3

Means and standard deviations of percent weight loss results

Appendix 4

Means and standard deviations of tensile strength results

Appendix 5

Means and standard deviations of percent ultimate elongation results

APPENDIX ONE

MEDIA

All quantities shown are given in w/v basis. All reagents are standard laboratory grade. All media, unless indicated, were steam sterilized at 121°C for 15 minutes.

a. Malt extract agar (Oxoid CM 59) (MEA)

Malt extract	30.0g
Mycological peptone	5.0g
Agar No. 1	15.0g
Distilled water	1 litre
pH	5.4

Sterilized by autoclaving at 115°C for 10 minutes.

b. Czapek Dox agar (Oxoid CM 97) (CZA)

$NaNO_3$	2.0g
KCl	0.5g
Magnesium glycerophosphate	0.5g
$FeSO_4\ 7H_2O$	0.01g
K_2SO_4	0.35g
Sucrose	30.0g
Oxoid agar No. 3	12.0g
Distilled water	1 litre
pH	6.8

Sterilized by autoclaving at 115°C for 20 minutes

c. Cellulose agar (Eggins and Pugh, 1962)(CA)

KH_2PO_4	1.0g
$(NH_4)_2SO_4$	0.5g
KCl	0.5g
L-asparagine	0.5g
Yeast extract (Oxoid)	0.5g
$MgSO_4$ $7H_2O$	0.2g
$CaCl_2$	0.1g
Agar No. 1	20.0g
4% ball milled cellulose	250 ml
Distilled water	750 ml
pH	6.2

d. Malt extract agar + Aureomycin

After steam sterilizing malt extract agar (Oxoid CM 59), it was allowed to cool to about 45°C and 60mg/litre medium of membrane-filtered Aureomycin was added and mixed thoroughly.

e. Malt extract broth (Oxoid CM 57)

Malt extract	17.0g
Mycological peptone	3.0g
Distilled water	1 litre
pH	5.4

Sterilized by autoclaving at 115°C for 10 minutes.

f. FA No. 5 mineral salts medium (Berk et al., 1957) (NSM)

NH_4NO_3	1.0g
KH_2PO_4	0.7g
K_2HPO_4	0.7g
$MgSO_4\ 7H_2O$	0.7g
NaCl	0.005g
$FeSO_4\ 7H_2O$	0.002g
$ZnSO_4\ 7H_2O$	0.002g
$MnSO_4\ 4H_2O$	0.001g
Agar No. 1	20.0g
Distilled water	1 litre
pH	6.5

g. Starch agar (SA)

Mycological peptone	5.0g
Lab lemco powder	3.0g
Soluble starch	2.0g
Agar No. 1	15.0g
Distilled water	1 litre
pH	5.8

h. Gelatin agar (GA)

Mycological peptone	5.0g
Lab lemco powder	3.0g
Gelatin	4.0g
Agar No. 1	15.0g
Distilled water	1 litre
pH	5.4

An 8% gelatin solution in water was sterilized separately and added to the remainder of the sterilized medium at the rate of 5ml per 100ml medium.

i. Polyester agar (PEA)

Mycological peptone	5.0g
Lab lemco powder	3.0g
Polyester (ethylene glycol/butane diol 50:50 adipate)	5.0g
Agar No. 1	15.0g
Distilled water	1 litre
pH	5.6

j. Urea agar (UA)

Malt extract	10.0g
Urea	5.0g
NaH_2PO_4 $2H_2O$	1.3682g
Na_2HPO_4 $12H_2O$	0.4406g
Bromothymol blue (2%)	12 ml
Distilled water	1 litre
pH	5.7

A urea solution of 0.5g per ml was prepared and sterilized by membrane filtration. One ml of the sterile urea solution was added to each 100ml of the previously sterilized and cooled (about 45°C) remainder of the medium.

k. Urea broth

The above medium (j) was prepared without agar according to the same procedure.

l. Basal medium (PLA)

Mycological peptone	5.0g
Lab lemco powder	3.0g
Agar	15.0g
Distilled water	1 litre
pH	5.7

APPENDIX TWO

POLYURETHANE ELASTOMERS

	Shore A hardness
Yellow soft polyester polyurethane elastomer	55
Red medium polyester polyurethane elastomer	60
White hard polyester polyurethane elastomer	90
Red hard polyester polyurethane elastomer	95
Yellow hard polyether polyurethane elastomer	90

APPENDIX THREE

MEANS AND STANDARD DEVIATIONS OF PERCENT WEIGHT LOSS RESULTS

FUNGUS	AGAR MEDIUM	PERCENT WEIGHT LOSS			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	0.0 ± 0.0	3.00 ± 0.32	3.74 ± 0.21	12.43 ± 2.16
	PLA	0.0 ± 0.0	7.73 ± 1.78	13.59 ± 1.20	18.23 ± 0.40
C. globosum	MEA	0.0 ± 0.0	1.19 ± 0.20	4.56 ± 0.37	15.19 ± 1.57
	PLA	0.0 ± 0.0	5.33 ± 1.01	5.94 ± 0.37	9.25 ± 0.57
P. citrinum	MEA	0.0 ± 0.0	16.01 ± 2.20	25.11 ± 0.92	38.78 ± 1.23
	PLA	0.0 ± 0.0	35.90 ± 2.28	45.05 ± 4.52	67.62 ± 5.29
A. fumigatus	MEA	0.0 ± 0.0	2.46 ± 0.35	2.97 ± 0.68	22.61 ± 1.32
	PLA	0.0 ± 0.0	4.33 ± 0.21	5.17 ± 0.77	14.24 ± 3.67

TABLE 5.4

PERCENT WEIGHT LOSS MEASUREMENTS OF YELLOW SOFT POLYESTER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	PERCENT WEIGHT LOSS			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	0.0 ± 0.0	2.51 ± 0.16	3.00 ± 0.38	11.58 ± 0.85
	PLA	0.0 ± 0.0	7.94 ± 0.71	14.24 ± 0.62	16.40 ± 1.37
C. globosum	MEA	0.0 ± 0.0	1.17 ± 0.15	4.06 ± 0.48	15.25 ± 0.89
	PLA	0.0 ± 0.0	8.62 ± 0.97	9.71 ± 1.64	14.51 ± 0.98
P. citrinum	MEA	0.0 ± 0.0	12.29 ± 0.44	24.61 ± 2.90	42.06 ± 5.10
	PLA	0.0 ± 0.0	34.70 ± 2.94	44.86 ± 2.75	63.76 ± 2.67
A. fumigatus	MEA	0.0 ± 0.0	2.35 ± 0.53	2.20 ± 0.25	19.48 ± 1.66
	PLA	0.0 ± 0.0	5.63 ± 0.46	8.90 ± 0.77	27.09 ± 1.35

TABLE 5.5

PERCENT WEIGHT LOSS MEASUREMENTS OF RED MEDIUM POLYESTER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	PERCENT WEIGHT LOSS			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	0.0 ± 0.0	2.77 ± 0.26	3.26 ± 0.30	12.67 ± 0.60
	PLA	0.0 ± 0.0	16.46 ± 1.03	18.92 ± 0.96	25.49 ± 5.68
C. globosum	MEA	0.0 ± 0.0	2.28 ± 0.65	6.57 ± 0.55	17.29 ± 1.05
	PLA	0.0 ± 0.0	5.61 ± 0.23	6.99 ± 0.27	10.27 ± 0.52
P. citrinum	MEA	0.0 ± 0.0	15.24 ± 2.01	28.02 ± 2.64	47.25 ± 2.73
	PLA	0.0 ± 0.0	38.97 ± 1.84	50.36 ± 3.66	64.52 ± 1.14
A. fumigatus	MEA	0.0 ± 0.0	4.17 ± 0.40	4.47 ± 0.63	33.01 ± 1.75
	PLA	0.0 ± 0.0	14.49 ± 1.91	28.16 ± 1.65	48.45 ± 1.57

TABLE 5.6

PERCENT WEIGHT LOSS MEASUREMENTS OF WHITE HARD POLYESTER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	PERCENT WEIGHT LOSS			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	0.0 ± 0.0	1.22 ± 0.43	2.32 ± 0.16	6.84 ± 0.74
	PLA	0.0 ± 0.0	5.09 ± 0.84	11.07 ± 3.33	13.77 ± 1.22
C. globosum	MEA	0.0 ± 0.0	1.04 ± 0.15	2.18 ± 0.59	7.68 ± 0.47
	PLA	0.0 ± 0.0	3.58 ± 0.15	3.87 ± 0.19	5.01 ± 0.13
P. citrinum	MEA	0.0 ± 0.0	10.59 ± 0.49	20.77 ± 2.19	36.59 ± 0.73
	PLA	0.0 ± 0.0	26.32 ± 1.76	37.75 ± 0.58	52.97 ± 2.84
A. fumigatus	MEA	0.0 ± 0.0	1.76 ± 0.82	2.73 ± 0.18	15.02 ± 0.95
	PLA	0.0 ± 0.0	8.28 ± 0.76	15.48 ± 1.03	20.18 ± 1.01

TABLE 5.7

PERCENT WEIGHT LOSS MEASUREMENTS OF RED HARD POLYESTER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	PERCENT WEIGHT LOSS			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	0.0 ± 0.0	0.23 ± 0.06	0.25 ± 0.04	0.66 ± 0.04
	PLA	0.0 ± 0.0	0.25 ± 0.05	0.42 ± 0.05	0.77 ± 0.07
C. globosum	MEA	0.0 ± 0.0	-0.47 ± 0.07	-0.69 ± 0.32	-0.62 ± 0.19
	PLA	0.0 ± 0.0	0.32 ± 0.03	0.37 ± 0.08	0.74 ± 0.07
P. citrinum	MEA	0.0 ± 0.0	0.28 ± 0.04	0.29 ± 0.05	0.41 ± 0.07
	PLA	0.0 ± 0.0	0.38 ± 0.05	0.42 ± 0.04	0.68 ± 0.07
A. fumigatus	MEA	0.0 ± 0.0	0.44 ± 0.03	0.29 ± 0.10	0.53 ± 0.07
	PLA	0.0 ± 0.0	0.40 ± 0.03	0.42 ± 0.04	0.68 ± 0.07

TABLE 5.8

PERCENT WEIGHT LOSS MEASUREMENTS OF YELLOW HARD POLYETHER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

APPENDIX FOUR

MEANS AND STANDARD DEVIATIONS OF TENSILE STRENGTH RESULTS

TABLE 6.5

TENSILE STRENGTH MEASUREMENTS OF YELLOW SOFT POLYESTER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	TENSILE STRENGTH (g/mm^2)			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	6195 ± 318	2261 ± 446	334 ± 69	114 ± 30
	PLA	6195 ± 318	712 ± 135	481 ± 88	227 ± 21
C. globosum	MEA	6195 ± 318	5864 ± 445	4136 ± 457	1618 ± 135
	PLA	6195 ± 318	2369 ± 344	1768 ± 177	1371 ± 144
P. citrinum	MEA	6195 ± 318	2572 ± 300	518 ± 150	239 ± 101
	PLA	6195 ± 318	244 ± 188	85 ± 68	0 ± 0
A. fumigatus	MEA	6195 ± 318	1403 ± 256	422 ± 121	0 ± 0
	PLA	6195 ± 318	459 ± 120	213 ± 40	176 ± 45

TABLE 6.6

TENSILE STRENGTH MEASUREMENTS OF RED MEDIUM POLYESTER POLYURETHANES AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	TENSILE STRENGTH (g/mm^2)			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	7001 ± 318	2611 ± 190	325 ± 40	101 ± 22
	PLA	7001 ± 318	817 ± 138	396 ± 117	236 ± 28
C. globosum	MEA	7001 ± 318	6236 ± 250	4477 ± 506	2160 ± 161
	PLA	7001 ± 318	5765 ± 438	3242 ± 566	1234 ± 212
P. citrinum	MEA	7001 ± 318	2350 ± 314	33 ± 27	0 ± 0
	PLA	7001 ± 318	108 ± 105	16 ± 16	0 ± 0
A. fumigatus	MEA	7001 ± 318	1480 ± 184	139 ± 88	36 ± 11
	PLA	7001 ± 318	227 ± 40	190 ± 66	0 ± 0

TABLE 6.7

TENSILE STRENGTH MEASUREMENTS OF WHITE HARD POLYESTER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	TENSILE STRENGTH (g/mm^2)			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	6589 ± 285	2675 ± 233	1115 ± 328	378 ± 107
	PLA	6589 ± 285	541 ± 111	441 ± 94	103 ± 30
C. globosum	MEA	6589 ± 285	6141 ± 543	4512 ± 249	1922 ± 277
	PLA	6589 ± 285	4639 ± 151	3438 ± 168	2197 ± 103
P. citrinum	MEA	6589 ± 285	2470 ± 375	1280 ± 407	0 ± 0
	PLA	6589 ± 285	197 ± 115	89 ± 79	0 ± 0
A. fumigatus	MEA	6589 ± 285	3109 ± 245	1096 ± 421	89 ± 64
	PLA	6589 ± 285	634 ± 96	197 ± 97	0 ± 0

TABLE 6.8

TENSILE STRENGTH MEASUREMENTS OF RED HARD POLYESTER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	TENSILE STRENGTH (g/mm^2)			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	5894 ± 311	1991 ± 216	1121 ± 247	526 ± 130
	PLA	5894 ± 311	1130 ± 190	513 ± 260	0 ± 0
C. globosum	MEA	5894 ± 311	5768 ± 446	5111 ± 331	3626 ± 201
	PLA	5894 ± 311	5016 ± 269	4454 ± 210	4056 ± 254
P. citrinum	MEA	5894 ± 311	3427 ± 39	1784 ± 240	69 ± 58
	PLA	5894 ± 311	587 ± 242	0 ± 0	0 ± 0
A. fumigatus	MEA	5894 ± 311	2298 ± 257	775 ± 192	0 ± 0
	PLA	5894 ± 311	243 ± 117	0 ± 0	0 ± 0

TABLE 6.9

TENSILE STRENGTH MEASUREMENTS OF YELLOW HARD POLYETHER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	TENSILE STRENGTH (g/mm^2)			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	5499 ± 272	5139 ± 541	5394 ± 207	5269 ± 229
	PLA	5499 ± 272	5218 ± 381	5125 ± 203	4813 ± 281
C. globosum	MEA	5499 ± 272	5240 ± 318	5365 ± 386	5088 ± 168
	PLA	5499 ± 272	5184 ± 327	4955 ± 369	4897 ± 278
P. cintrinum	MEA	5499 ± 272	5047 ± 480	5080 ± 380	5302 ± 331
	PLA	5499 ± 272	5221 ± 289	5287 ± 307	4978 ± 432
A. fumigatus	MEA	5499 ± 272	5148 ± 256	4534 ± 373	5076 ± 79
	PLA	5499 ± 272	5170 ± 341	5106 ± 274	4975 ± 242

APPENDIX FIVE

MEANS AND STANDARD DEVIATIONS OF PERCENT ULTIMATE ELONGATION RESULTS

FUNGUS	AGAR MEDIUM	PERCENT ULTIMATE ELONGATION			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	1155.4 ± 21.8	897.5 ± 46.1	235.7 ± 67.3	35.3 ± 12.4
	PLA	1155.4 ± 21.8	533.4 ± 63.4	403.6 ± 68.6	173.6 ± 32.3
C. globosum	MEA	1155.4 ± 21.8	1148.6 ± 25.7	1062.6 ± 71.3	838.2 ± 24.4
	PLA	1155.4 ± 21.8	927.1 ± 36.2	850.9 ± 28.4	812.5 ± 51.0
P. citrinum	MEA	1155.4 ± 21.8	973.7 ± 27.7	595.5 ± 84.3	213.7 ± 84.1
	PLA	1155.4 ± 21.8	259.6 ± 94.5	115.9 ± 80.7	0.0 ± 0.0
A. fumigatus	MEA	1155.4 ± 21.8	783.2 ± 51.4	341.5 ± 96.8	0.0 ± 0.0
	PLA	1155.4 ± 21.8	366.9 ± 87.0	127.0 ± 69.0	94.9 ± 36.8

TABLE 6.10

PERCENT ULTIMATE ELONGATION MEASUREMENTS OF YELLOW SOFT POLYESTER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	PERCENT ULTIMATE ELONGATION			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	1155.8 ± 23.2	879.1 ± 23.7	144.0 ± 37.9	24.0 ± 5.8
	PLA	1155.8 ± 23.2	513.1 ± 56.3	327.4 ± 73.1	87.5 ± 18.7
C. globosum	MEA	1155.8 ± 23.2	1143.0 ± 28.1	1031.5 ± 40.1	867.8 ± 20.6
	PLA	1155.8 ± 23.2	951.1 ± 25.3	969.4 ± 33.8	726.7 ± 52.1
P. citrinum	MEA	1155.8 ± 23.2	862.2 ± 44.4	36.7 ± 36.6	0.0 ± 0.0
	PLA	1155.8 ± 23.2	134.0 ± 90.7	4.2 ± 3.8	0.0 ± 0.0
A. fumigatus	MEA	1155.8 ± 23.2	721.1 ± 40.7	64.9 ± 49.3	48.0 ± 11.6
	PLA	1155.8 ± 23.2	131.2 ± 75.2	88.9 ± 28.8	0.0 ± 0.0

TABLE 6.11

PERCENT ULTIMATE ELONGATION MEASUREMENTS OF RED MEDIUM POLYESTER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	PERCENT ULTIMATE ELONGATION			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	1102.8 ± 29.2	691.5 ± 27.9	416.3 ± 86.8	134.6 ± 44.9
	PLA	1102.8 ± 29.2	317.5 ± 53.9	291.5 ± 48.6	75.0 ± 51.8
C. globosum	MEA	1102.8 ± 29.2	1088.0 ± 52.1	989.2 ± 39.2	714.0 ± 59.6
	PLA	1102.8 ± 29.2	959.0 ± 17.4	855.1 ± 13.8	670.3 ± 15.8
P. citrinum	MEA	1102.8 ± 29.2	757.8 ± 42.3	637.8 ± 87.1	0.0 ± 0.0
	PLA	1102.8 ± 29.2	352.0 ± 63.7	265.1 ±100.7	0.0 ± 0.0
A. fumigatus	MEA	1102.8 ± 29.2	808.6 ± 27.1	421.9 ± 77.8	59.3 ± 46.4
	PLA	1102.8 ± 29.2	320.3 ± 42.7	90.3 ± 68.2	0.0 ± 0.0

TABLE 6.12

PERCENT ULTIMATE ELONGATION MEASUREMENTS OF WHITE HARD POLYESTER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	PERCENT ULTIMATE ELONGATION			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	884.6 ± 26.7	313.3 ± 33.5	203.2 ± 65.8	53.6 ± 33.4
	PLA	884.6 ± 26.7	157.1 ± 45.8	77.6 ± 59.5	0.0 ± 0.0
C. globosum	MEA	884.6 ± 26.7	859.3 ± 52.6	793.0 ± 42.5	632.2 ± 29.2
	PLA	884.6 ± 26.7	791.5 ± 28.7	705.6 ± 33.8	637.8 ± 38.7
P. citrinum	MEA	884.6 ± 26.7	661.8 ± 16.5	526.4 ± 31.4	14.1 ± 10.6
	PLA	884.6 ± 26.7	390.1 ± 57.4	0.0 ± 0.0	0.0 ± 0.0
A. fumigatus	MEA	884.6 ± 26.7	342.9 ± 37.5	161.7 ± 36.1	0.0 ± 0.0
	PLA	884.6 ± 26.7	45.15 ± 16.7	0.0 ± 0.0	0.0 ± 0.0

TABLE 6.13

PERCENT ULTIMATE ELONGATION MEASUREMENTS OF RED HARD POLYESTER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

FUNGUS	AGAR MEDIUM	PERCENT ULTIMATE ELONGATION			
		0 WEEKS	1 WEEK	2 WEEKS	4 WEEKS
G. roseum	MEA	849.3 ± 18.7	842.4 ± 31.2	850.9 ± 7.9	850.9 ± 18.2
	PLA	849.3 ± 18.7	824.1 ± 16.0	838.2 ± 9.8	828.3 ± 15.0
C. globosum	MEA	849.3 ± 18.7	841.0 ± 12.6	842.4 ± 14.5	852.3 ± 8.0
	PLA	849.3 ± 18.7	831.4 ± 18.1	831.1 ± 23.8	838.6 ± 13.6
P. citrinum	MEA	849.3 ± 18.7	835.4 ± 21.7	836.8 ± 24.2	850.9 ± 25.3
	PLA	849.3 ± 18.7	838.2 ± 18.3	855.2 ± 19.6	849.5 ± 28.8
A. fumigatus	MEA	849.3 ± 18.7	845.4 ± 7.5	822.7 ± 38.3	853.7 ± 11.4
	PLA	849.3 ± 18.7	841.0 ± 19.4	848.1 ± 15.0	846.7 ± 17.6

TABLE 6.14

PERCENT ULTIMATE ELONGATION MEASUREMENTS OF YELLOW HARD POLYETHER POLYURETHANE AFTER INCUBATION UP TO 4 WEEKS WITH TEST FUNGI ON MEA AND PLA MEDIA

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